

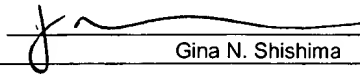


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Gina N. Shishima

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Zhang et al.

Serial No.: 09/203,078

Filed: December 1, 1998

For: METHOD FOR THE PRODUCTION AND
PURIFICATION OF ADENOVIRAL
VECTORS

Group Art Unit: 1648

Examiner: Foley, Shanon A.

Atty. Dkt. No.: INRP:081US

APPEAL BRIEF

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PATENT

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APPEAL BRIEF

MS Appeal Brief

Commissioner for Patents
P. O. Box 1450
Alexandria, VA 22313-1450

Sir:

Appellants hereby submit this Appeal Brief to the Board of Patent Appeals and Interferences in response to the final Office Action dated November 17, 2004. The Notice of Appeal was filed on February 17, 2005 and received by the Patent and Trademark Office on February 22, 2005, as indicated on the stamped return postcard. Therefore, the deadline to file this Appeal Brief was April 22, 2005. A request for a three-month extension of time to respond is included herewith along with the required fee. This extension will bring the due date to July 22, 2005, which is within the six-month statutory period. Should such request or fee be deficient or absent, consider this paragraph such a request and authorization to withdraw the appropriate fee under 37 C.F.R. §§ 1.16 to 1.21 from Fulbright & Jaworski L.L.P. Account No.: 50-1212/INRP:081US.

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The fee for filing this Appeal Brief is \$250, and is attached hereto. If the check is inadvertently omitted, or should any additional fees under 37 C.F.R. §§ 1.16 to 1.21 be required for any reason relating to the enclosed material, or should an overpayment be included herein, the Commissioner is authorized to deduct or credit said fees from or to Fulbright & Jaworski L.L.P. Account No.: 50-1212/INRP:081US.

I. REAL PARTY IN INTEREST

The real party in interest is Introgen Therapeutics, Inc. in Austin, Texas.

II. RELATED APPEALS AND INTERFERENCES

No related appeals or interferences are presently pending.

III. STATUS OF THE CLAIMS

Claims 1-43 were originally filed on December 01, 1998.

In response to a Restriction Requirement Dated March 20, 2001, Appellants filed an amendment in which claims 1-29 were elected for prosecution and claims 30-43 were cancelled.

Claims 1-29 were rejected in the Office Action Dated September 13, 2001. In response to the Office Action Dated September 13, 2001, Appellants filed an amendment in which claims 3, 18, 23, 24, and 25 were amended

Claims 1-29 were rejected in the Office Action Dated October 3, 2003. In response to the Office Action Dated October, 3, 2003, Appellants filed an amendment in which claims 1, 2, 3, 18, and 29 were amended and claims 30-62 were added.

Claims 33-37 were withdrawn from consideration and claims 1-32 and claims 38-62 were rejected in the Office Action Dated June 3, 2004. In response to the Office Action Dated June 3, 2004, Appellants filed an amendment in which claim 32 was cancelled and claim 48 was amended.

Claims 1-31 and 33-62 were pending in the final Office Action Dated November 17, 2004. Thus claims 1-31 and 33-62 are pending on appeal and are the subject of this appeal brief.

IV. STATUS OF AMENDMENTS

No amendments have been filed subsequent to the final rejection.

V. SUMMARY OF CLAIMED SUBJECT MATTER

Claim 1 concerns a process for preparing adenovirus by preparing a culture of producer cells by seeding producer cells into a culture medium, infecting cells in the culture after they have reached a mid-log phase growth with a selected adenovirus, and harvesting the adenovirus particles from the cell culture. Specification at page 4, lines 9-19.

Claim 47 is a method for producing adenovirus that includes culturing producer cells and infecting the cultured producer cells with an adenovirus, wherein the cells in culture are between a mid-log phase of growth and a stationary phase of growth. Specification at page 4, line 16 to page 5, line 9.

Claim 3 concerns a preferred embodiment of the present invention, where the producer cells are seeded into the cell culture medium using an essentially homogeneous pool of cells. Specification at page 4, lines 11-20.

Claim 8 relates to seeding the cells in the culture medium so that they attach to the culture surface for between 3 and 24 hours prior to infection. Specification page 6, lines 20-27.

Claim 9 relates to a process where the culture medium is at least partially recirculated during the adenovirus infection step. Specification page 6, lines 14-18.

Claim 13 relates to a process where the harvested adenovirus is subjected to purification and placed in a pharmaceutically acceptable composition. Specification page 7, lines 14-27.

Claim 29 relates to a process where adenovirus having one or more properties of a) a virus titer of between about 1×10^9 and about 1×10^{13} pfu/ml, b) a virus particle concentration between about 1×10^{10} and about 2×10^{13} particles/ml, c) a particle:pfu ratio between about 10 and about 60, d) having less than 50 ng BSA per 1×10^{12} viral particles, e) between about 50 pg and 1 ng of contaminating human DNA per 1×10^{12} viral particles, f) a single HPLC elution

peak consisting essentially of 97 to 99% of the area under the peak. Specification page 9, line 10 to page 10, line 15.

Claim 31 also relates to putting a virus having one or more of the properties of claim 29 into a pharmaceutically acceptable composition. Specification page 7, lines 14-27.

Claim 58 relates to the method of 47 where the producer cells are essentially homogenous with phase of cell growth. Specification at page 4, lines 11-20.

VI. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

1) Are claims 1, 3, 8, 9, 13-25, 31, 38, 47, 49 and 52-62 properly rejected as anticipated by Huyghe *et al.*, *Human Gene Therapy* 6:1403-1416 (1995) (“Huyghe”) (Exhibit 1) in light of Robert J. Kuchler, *Biochemical Methods in Cell Culture and Virology* (1977) (“Kuchler”) (Exhibit 4) under 35 U.S.C. § 102(b)?

2) Are claims 1, 3-9, 13-28, 30, 31, 38-49 and 51-62 properly rejected as anticipated by Zhang *et al.* (WO 98/22588) (“Zhang PCT”) (Exhibit 6) as further evidenced by Wu *et al.* (U.S. Patent 6,689,600 B1) (“Wu”) (Exhibit 7) under 35 U.S.C. § 102(a)?

3) Are claims 1, 3-9, 13-28, 30, 31, 38-49 and 51-62 properly rejected as anticipated by Zhang *et al.* (U.S. Patent 6,194,191) (“Zhang Patent”) (Exhibit 9) as further evidenced by Wu under 35 U.S.C. § 102(e)?

4) Are claims 10-12 and 29 properly rejected as being unpatentable over Huyghe as applied to claims 1, 3, 8, 9, 13-25, 31, 38, 47, 49 and 51-62 above or the Zhang PCT or Zhang Patent (collectively “Zhang references”) as applied to claims 1, 3-9, 13-28, 30, 31, 38-49, and 51-62 under 35 U.S.C. § 103(a)?

5) Are claims 2 and 50 properly rejected as being unpatentable over Huyghe as applied to claims 1, 3, 8, 9-12, 13-25, 29, 31, 38, 47, 49, and 51-62 above or Zhang references as applied to claims 1, 3-31, 38-49 and 51-62 and further in view of Graham and Prevec, *In*:

Methods in Molecular Biology: Gene Transfer and Expression Protocols 7 (“Graham”) (Exhibit 10) and Leu *et al.* (U.S. Patent 6,194,210 B1) (“Leu”) (Exhibit 11) under 35 U.S.C. § 103(a)?

6) Are claims 26-28 properly rejected as being unpatentable over Huyghe as applied to claims 1, 3, 8, 9-12, 13-25, 29, 31, 38, 47, 49, and 51-62 above and further in view of Graham (Exhibit 10) under 35 U.S.C. § 103(a)?

7) Are claims 4, 30, 39-46 and 48 properly rejected as being unpatentable over Huyghe as applied to claims 1, 3, 8, 9-12, 13-25, 29, 31, 38, 47, 49 and 51-62 above, and further in view of Garnier *et al.*, *Cytotechnol.*, 15:145-155, 1994, (“Garnier”) (Exhibit 12) and Spier, R.E. and J.B. Griffiths, eds., *Animal Cell Biotechnology*, Vol. 3 (1988), (“Spier”) (Exhibit 13) under 35 U.S.C. § 103(a)?¹

VII. ARGUMENT

A. Substantial Evidence Required to Uphold the Examiner’s Position

As an initial matter, Appellants note that findings of fact and conclusions of law by the U.S. Patent and Trademark Office must be made in accordance with the Administrative Procedure Act, 5 U.S.C. § 706(A), (E), 1994. *Dickinson v. Zurko*, 527 U.S. 150, 158 (1999). Moreover, the Federal Circuit has held that findings of fact by the Board of Patent Appeals and Interferences must be supported by “substantial evidence” within the record. *In re Gartside*, 203 F.3d 1305, 1315 (Fed. Cir. 2000). In *Gartside*, the Federal Circuit stated that “the ‘substantial

¹ The Action provisionally rejects claims 1, 3-29, 38, 47-62 under the judicially created doctrine of obviousness-type double patenting over claims 1-29 of U.S. Publication No. 2002/018723 (U.S. Application Ser. No. 09/880,609). Appellants note that the claims now pending in the ’609 prosecution are believed to be patentably distinct from the present case. In any event, because this rejection is provisional, Appellants will address the rejection once claims in either application are deemed in condition for allowance.

evidence' standard asks whether a reasonable fact finder could have arrived at the agency's decision." *Id.* at 1312.

Accordingly, it necessarily follows that an Examiner's position on Appeal must be supported by "substantial evidence" within the record in order to be upheld by the Board of Patent Appeals and Interferences.

B. The Burden of Proof to Establish Inherent Anticipation of Claims 1, 3, 8, 9, 13-25, 31, 38, 47, 49, and 52-62 by Huyghe in Light of Kuchler Has Not Been Met

The Examiner has not met the burden of proof to establish inherent anticipation of independent claims 1 and 47 by Huyghe in light of Kuchler under 35 U.S.C. § 102(b) because the Examiner has neither shown that the characteristics of the present invention are necessarily present in the prior art, nor provided a basis to reasonably support such a determination. "Anticipation by inherency requires that 1) the missing descriptive matter be 'necessarily present' in the prior art reference and that 2) it would be so recognized by persons of ordinary skill in the art." *Continental Can Co. v. Monsanto Co.*, 948 F.2d 1264, 1268, 20 U.S.P.Q.2d 1746, 1749 (Fed. Cir. 1991). "In relying upon the theory of inherency, the examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (Bd. Pat. App. & Inter. 1990). Here, the Examiner relies on specific data from a behaviorally and genetically dissimilar cell type, fails to connect this specific data to the cells of the present invention, and yet still determines, incorrectly, that inherent anticipation exists.

1. Background of the Dispute

Claim 1 and 47 of the present invention relate to a process and a method, respectively, for producing adenovirus, where producer cells are cultured and then infected when the cells are

between mid-log and stationary phase of growth. The prior art reference Huyghe (Exhibit 1) teaches infecting producer cell cultures with adenovirus when the cultures are between 50% and 60% confluent.

In the Office Action Dated September 13, 2001 (Exhibit 14), several claims, including claim 1, were rejected under 35 U.S.C. § 102(b) as being anticipated by Huyghe because the Examiner confused phases of the cell cycle (M, G₁, S, and G₂), with phases of a cell population growth curve (lag phase, log phase, and stationary phase). “Since the cells were infected at 50-60% confluency, the cell culture would inherently comprise cells at every phase in the cell cycle at the time of infection and therefore anticipate infecting cells at a particular phase.” Action at page 6, paragraph 4.

Appellants responded by submitting the February 13, 2002 Declaration of Shawn Gallagher Under 37 C.F.R. § 1.132 (Exhibit 2) (“Gallagher Declaration”). Mr. Gallagher pointed out the Examiner’s error, and then chose to provide additional information showing that were the rejection based on phase of cell growth instead phase of cell cycle, Huyghe still does not disclose the present invention. Mr. Gallagher indicated that with what little information Huyghe provided, there was no way to *actually conclude* what phase of growth the cells were in at the time of infection, but that it might be possible to estimate the phase of growth if one were to make certain assumptions and extrapolations.

In the Huyghe *et al.* reference at page 1404, in the second paragraph under “MATERIALS AND METHODS,” the article indicates that the cell monolayers were at “50-60% confluency” when infected with adenovirus. However, there is no information provided on the seeding density, lag phase or the doubling times for the 293 cells under the authors’ care, such that would permit a determination of whether these cells were in “mid-log” to “stationary growth” phases at the time they were infected – 2 to 2.5 days after seeding. Without knowledge of seeding density, lag phase or doubling times for the cells used in the study, there is no way one can conclude that Huyghe *et al.* infected the cells between mid-log and stationary phase.

Gallagher Declaration, paragraph 6.

At best, one can merely estimate the “phase of the culture described in the Huyghe *et al.* reference, making certain assumptions and extrapolations, when infected at 2-2.5 days and 50-60% confluency. If one assumes that Huyghe *et al.* seeded at a seeding density of approximately $1-3 \times 10^4$ cells/cm², and that the 293 cells employed by Huyghe *et al.* had a lag phase of approximately 24 hours and a doubling time of approximately 36 hours, then one can calculate the phase at 50-60% confluency as early log phase, certainly less than mid-log phase, using the following calculations:

- **Initial density (midpoint of assumption range) = 2×10^4 cells/cm²**
- **Growth period (2.5 days – lag time) = 1.5 days = 36 hours**
- **With a doubling time of 36 hours, the cell population will double once, giving final concentration equal to 2×10^4 cells/cm² $\times 2 = 4 \times 10^4$ cells/cm², consistent with early log phase density**

Gallagher Declaration, paragraph 7.

Mr. Gallagher then referenced publications to support his opinion that the cells of Huyghe were at the most, in early log phase. Among these are the MediaTech reference (Exhibit 3), which stands for the proposition that one should use *at least* 70% confluent cultures to ensure that they are in log phase, and the Kuchler reference indicating that the lag phase usually varies from 24 to 48 hours.

In MediaTech’s Technical Information Bulletin, it is indicated, in the first paragraph of column 2 on the first page, that in order to ensure that cultures are in log phase, they must be at least 70% confluent. Thus, a culture that is only 50-60% confluent like that of Huyghe *et al.* is likely not in log phase, or at the very least, only in early log phase, not mid-log or late-log phase.

Gallagher Declaration, paragraph 9.

Next, I refer to the article of Kuchler, “Biochemical Methods in Cell Culture and Virology.” At page 90, the lag phase, which precedes the log phase, is said to vary from 24 to 48 hours. Given that Huyghe *et al.* infected cells between 48-60 hours after seeding, Kuchler suggests that Huyghe *et al.*’s cells would be barely out of lag phase. While specific cell lines or strains of cell lines can demonstrate significant variation in characteristic doubling times and duration of lag phase, our own experience with 293 cells from various sources indicates that lag times of 24-48 hours are not uncommon after passaging.

Gallagher Declaration , paragraph 10.

2. Basis of Current Rejection

Independent claims 1 and 47 are now rejected under 35 U.S.C. § 102(b) as being anticipated by Huyghe in light of Kuchler. The Examiner contends that the cells of Huyghe are at mid-phase upon infection based on the following: “1) lag time of 293 cells ranges between 2—48 hours, 2) the cells of Huyghe have a confluency of 50-60% upon infection, 3) the cells of Huyghe attach to the surface of the plate for 48-60 hours before infection, which is beyond lag phase time, and 4) the chart provided by Kuchler indicates that the growth curve of cells after 60 hours of incubation is the mid-point of the growth curve, *i.e.*, mid-phase.” (Office Action, June 3, 2004, page 7, paragraph 2) (Exhibit 15). This same rejection is maintained for reasons of record in the Office Action Dated November 17, 2004 (page 6, paragraph 2) (Exhibit 16). As explained below, reliance on the Kuchler chart is inappropriate.

3. The Examiner has not met the burden of proof to establish inherent anticipation because the Kuchler chart does not provide a basis to reasonably support this determination.

Based on the chart provided by Kuchler, the Examiner cannot reasonably support a determination that Huyghe infected 293 cells at mid-log. In Huyghe, *human* 293 cells *attach* to the surface of the plate before infection. The chart provided by Kuchler (presumably 3-1), shows the growth curve of *mouse* L-M fibroblasts grown in *suspension*. The Examiner has not identified any connection between L-M fibroblast cells in suspension and 293 cells grown on plates, stating only that “since the general teachings of Kuchler relied upon by the applicant as applying to the cells of Huyghe *et al.* is relevant, it is determined that all the general teachings of Kuchler are relevant.” (November 17, 2004 Office Action, page 5, paragraph 2). The explanation given by the Examiner is flawed. If the Examiner must provide objective evidence (factual or technical) to reasonably support a determination of inherency, then Examiner must provide objective evidence to support applying the growth curve of L-M fibroblasts in

suspension to the cells taught by Huyghe. Instead, the Examiner has not shown where Kuchler teaches that the growth curve data of L-M fibroblasts in a suspension culture should be used as the standard to determine the particular phase of growth for *any* mammalian cell in *any* culture system.

Alternatively, even if Kuchler were relevant to what Huyghe teaches, it appears that in the chart relied upon by the Examiner that the growth curve shows the cells to be slightly *before* mid-log phase at 60 hours. This chart does not provide evidence that the cells of Huyghe were infected after mid-log phase of growth.

Still further, it appears as though the L-M cells grown in suspension culture had a doubling time of somewhat less than 24 hours (approx. 2.5×10^5 cells at 24 hours; approx. 5.6×10^5 cells at 48 hours; approx. 8.5×10^5 at 60 hours; approx. 1.8×10^6 at 84 hours; which works out to about a 16 to 20 hrs doubling time). A 16-20 hour doubling time would mean that the L-M cells would be expected to arrive at mid-log much quicker than a cell having a doubling time of 36 hours as assumed by Mr. Gallagher for his 293 cell calculations (Gallagher Declaration, paragraph 7). The doubling time for 293 is consistent with that measured at the assignee of the present application, Introgen Therapeutics, Inc. *See* Second Declaration of Dr. Shuyuan Zhang under 37 C.F.R. § 1.132, dated September 3, 2004 (“Second Zhang Declaration”) (showing a doubling time of approx. 30 hours for 293 cells in T-150 flasks) (Exhibit 8). Assuming a doubling time of 30 hours as compared to the 16-20 hour doubling time shown in Kuchler’s Figure 1-3, the growth curve of Figure 1-3 would be well below mid-log.

By not establishing how growth curve data of L-M fibroblasts grown in suspension culture relate to the 293 cells grown on plates in Huyghe, if there is any relation at all, one cannot establish the time of mid-log. By not establishing the time of mid-log of the 293 cells in

Huyghe, one cannot establish the phase of the cells at the time of infection. Because the Examiner cannot establish the phase of the cells taught by Huyghe at the time of infection, the Examiner cannot reasonably support the assertion that the cells of Huyghe were necessarily between mid-log and stationary phase of growth at the time of infection. As such, the Examiner has not met the burden of proof to establish inherent anticipation of claims 1 and 47 of the present invention by Huyghe.

4. Seeding density is a crucial component of log phase and Huyghe is silent as to seeding density

The examiner, without first meeting the burden of proof to establish inherent anticipation, nevertheless demands the calculations and the MediaTech reference provided by Mr. Gallagher be supported and substantiated (June 3, 2004 Office Action, page 6, paragraph 2, page 7 paragraph 1). In other words, the Appellants are asked to disprove the Examiner's unproven assertions.

The Examiner objects to the Mr. Gallagher's calculations, stating "Since seeding density is established in the art as a crucial component of log phase and Huyghe *et al.* has not provided any information regarding the initial seeding density of cells, [appellant's] presumption of early log phase density for the cells of Huyghe *et al.* is speculative and unsubstantiated." (Office Action Dated June 3, 2004, page 6, paragraph 2). First, it must be noted that the burden has not been properly shifted to the Appellants to provide absolutely substantiated evidence. Second, if the Examiner admits that seeding density is crucial, and due to the absence of information regarding seeding density any conclusion drawn from Huyghe would be "speculative and unsubstantiated," it can only be concluded that the Examiner has failed to make a *prima facie* case of anticipation. *In re Robertson*, 169 F.3d 743, 785 (Fed. Cir. 1999) (stating that

anticipation “may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient.”)

Additionally, in referring to Mediatech’s Technical Information in the Office Action Dated June 3, 2004, the Examiner states that Mr. Gallagher’s conclusion that 50-60% confluency equates to early log phase is unsupported in that Mediatech simply states that 70% confluent cultures are in log phase and “there is no differentiation between the various stages of log growth and % confluency provided by” the reference (June 3, 2004 Office Action, page 7, paragraph 1). Examiner relies on the MediaTech reference to argue that because the reference does not differentiate between the various stages of cell culture growth at 70% confluency, the Appellants must show that the lower 50-60% confluency of cell cultures taught by Huyghe are *not* between mid-log and stationary phase of growth. While the Examiner mischaracterizes the reference, which implies, as stated by Mr. Gallagher, that cultures that are less than 70% cannot be assured of even being in log phase, it again must be noted that the burden has not been properly shifted to the Appellants.

For the foregoing reasons, Appellants contend that the Examiner has not met the burden of proof in establishing inherent anticipation of claims 1 and 47. The Examiner has not provided objective evidence to support applying the growth curve of L-M fibroblasts in suspension mentioned by Kuchler to the cells taught by Huyghe. Therefore, the Examiner *does not* have any basis that would reasonably support a determination of inherent anticipation - that the step of infecting cells between mid-log and stationary phase of growth was a “necessarily present” step in the infection process of Huyghe. Additionally, the Examiner insists that without crucial knowledge of seeding density any determination of whether the cells of Huyghe were in log phase at the time of infection would be speculative and unsubstantiated. Accordingly, the

Examiner cannot establish that the missing descriptive matter was necessarily present in the prior art reference. Therefore, Appellants respectfully request that the Examiner's rejections be reversed.

5. Dependent claims are not anticipated by Huyghe in Light of Kuchler

Dependent claims 3, 8, 9, 13-25, 31, 32, 38, 49 and 51-62 have been rejected by the Examiner as being anticipated by Huyghe in light of Kuchler in the June 3, 2004 Office Action, and maintained in the November 17, 2004 Office Action. For the following reasons, Appellants respectfully request that the Examiner's rejections be reversed.

Dependent claims 3, 8, 9, 13-25, 31, and 38, which derive from independent claim 1, and dependent claims 49 and 51-62 which derive from claim 47 are not anticipated by Huyghe in light of Kuchler, because independent claims 1 and 47 are not anticipated as discussed above. "A claim is anticipated only if each and every element as set forth in the claim is found either expressly or inherently described, in a single prior art reference." *Verdegall Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 U.S.P.Q.2d 1051, 1053 (Fed. Cir. 1987).

Claim 1 is directed to:

- [a] process for preparing adenovirus, the process comprising:
 - (a) preparing a culture of producer cells in a selected media;
 - (b) infecting producer cells in the culture with adenovirus, wherein the producer cells are infected between mid-log phase of growth and stationary phase of growth; and
 - (c) harvesting adenovirus from the cell culture.

Claim 47 is directed to:

- [a] method for producing adenovirus that includes culturing producer cells and infecting the cultured producer cells with an adenovirus, wherein the improvement comprises infecting said producer cells with the adenovirus when the cells in culture are between mid-log phase of growth and stationary phase of growth.

Because Huyghe does not disclose, either expressly or inherently, infecting producer cells with adenovirus when the producer cells are between mid-log and stationary phase of growth, claims which are dependent to claim 1 or 47 are not anticipated by Huyghe in light of Kuchler.

Dependent claims 3, 8, 9, 13, 31, and 59 include further limitations such that they are separately unanticipated by Huyghe in light of Kuchler

a) Claim 3

Claim 3 is directed to the process of claim 1, wherein the producer cells are essentially homogenous with respect to the phase of growth. In the Office Action Dated June 3, 2004 and maintained, in the Office Action Dated November 17, 2004, the Examiner argues that “Huyghe *et al.* anticipate preparing adenovirus by culture of 293 producer cells that have attained an essentially homogenous confluency of 50-60%” and that “this percentage of confluency reasonably corresponds to mid-log phase of cell growth” (Office Action Dated June 3, 2004, page 5, paragraph 4 to page 6, paragraph 1). However, the Examiner has not pointed out, and the Appellants have found, any mention “homogenous” producer cells mentioned in Huyghe. Therefore, Appellants respectfully request that the board reverse the Examiner’s rejection.

b) Claim 8

Claim 8 is directed to the process of claim 1 wherein the producer cells are seeded into the culture medium and allowed to attach to a culture surface for between about 3 hours and about 24 hours prior to infection with adenovirus. The Examiner has not argued that Huyghe teaches infection between 3 and 24 hours after attachment. Instead, the Examiner points out that the cells “are allowed to attach to the surface between 2 and 2.5 days prior to infection” (Office Action Dated June 3, 2004, page 6, paragraph 1). Thus, the Examiner indicates that the cells taught by Huyghe were allowed to attach to the surface a minimum of 48 hours prior to infection, as opposed to the about 3 to about 24 hours of claim 8. Because Huyghe does not expressly or

inherently describe each and every element as set forth in the claim, Appellants respectfully request that the Examiner's rejection be reversed.

c) Claim 9

Claim 9 is directed to the process of claim 1, wherein the culture medium is at least partially recirculated during the adenovirus infection step. The Examiner has not shown where Huyghe teaches a recirculation step. Instead, Huyghe teaches mixing virus with fresh media and introducing this formulation into the cell factory containing 50-60% confluent cells. Huyghe provides no indication as to whether the culture medium was recirculated. The Examiner apparently mistakes thoroughly mixing adenovirus and *fresh* media *prior* to cell infection with recirculating the *culture* media *during* infection. Because Huyghe does not expressly or inherently describe each and every element as set forth in the claim, Appellants respectfully request that the Examiner's rejection be reversed.

d) Claim 13

Claim 13 is directed to the process of claim 1, wherein the harvested adenovirus is subjected to purification and placed into a pharmaceutically acceptable composition. The Examiner contends that Huyghe teaches harvesting the adenovirus and added to phosphate buffered saline supplemented with 2% sucrose and 2 mM MgCl₂, a pharmaceutically acceptable carrier. However, the Examiner has not shown where Huyghe teaches that this solution is a pharmaceutically acceptable composition. Additionally, it appears that the Examiner has not shown that this adenovirus is purified before placement into the phosphate buffered saline supplemented with 2% sucrose and 2 mM MgCl₂, as is required of claim 13. Therefore, Appellants respectfully request that the Examiner's rejection be reversed.

e) Claim 31

Claim 31 is dependent on claim 29. Claim 29 is the process of claim 1 wherein the adenovirus has one or more of several purification properties. Claim 31, is the process of claim 29, further comprising formulating the purified adenovirus composition into a pharmaceutically acceptable composition. For the reasons previously stated above, Appellants respectfully request that the Examiner's rejection be reversed.

f) Claim 58

Claim 58 is directed to the method of claim 47, wherein the producer cells are essentially homogenous with respect to phase of cell growth. The reasons supporting a lack of anticipation of claim 3 are applicable for claim 58. Therefore, appellants respectfully request that the Examiners' rejection be reversed.

C. Claims 1, 3-9, 13-28, 30, 31, 38-49 and 51-62 Are Not Anticipated by the Zhang PCT As Further Evidenced by Wu Under 35 U.S.C. § 102(a) or by the Zhang Patent As Further Evidenced by Wu Under 35 U.S.C. § 102(e)

1. The Zhang PCT Reference is Not Proper Prior Art Because it is Not "By Others"

Claims 1, 3-9, 13-28, 30, 31, 38-49 and 51-62 are not anticipated by the Zhang PCT reference as further evidenced by Wu under 35 U.S.C. § 102(a) because the Zhang PCT is not proper prior art. The invention that was disclosed but not claimed in the Zhang PCT is not "by others" within the meaning of 35 U.S.C. § 102(a) because it was invented by the same inventive entity as the present application.

In re Magner, 133 USPQ 404 (Bd. Pat. App. & Inter. 1961) stands for the proposition that when a prior publication is authored by fewer than all the inventors of the present application, that prior publication is not "by another" when the invention described was actually conceived of, researched, and reduced to practice by the inventors of the present application.

Claims 1 and 47 of the present invention relate to a process and a method, respectively, of culturing producer cells and infecting the producer cells with adenovirus between mid-log and stationary phase of growth. Claims 3-9, 13-28, 30, 31 and 38-46 depend ultimately from claim 1 and claims 48, 49 and 52-62 depend ultimately from claim 47. These claims were rejected in the Office Action Dated June 3, 2004 and maintained in the Office Action Dated November 17, 2004. In the June Office Action, the Examiner states that the Zhang PCT anticipates “a process of preparing adenovirus by preparing a culture of producer cells essentially homogenous with respect to growth, infecting the producer cells with adenovirus between mid-log and stationary phase, and harvesting the adenovirus and placing the virus into a pharmaceutically acceptable composition.” Action at page 9. The Examiner notes that the Zhang PCT and the Zhang Patent (discussed later) are identical disclosures and both share a common inventor with the present application. As the Zhang PCT is not a U.S. patent application, the Examiner rejects the present application under 35 U.S.C. § 102(a) presumably as being invented “by others.” See *In re Katz*, 687 F.2d 450, 215 USPQ 14 (CCPA 1982) (discussing the term “others” in 35 U.S.C. 102(a) refers to any entity which is different from the inventive entity).

In response, the Appellants submitted the Second Zhang Declaration to demonstrate that the invention disclosed but not claimed in the Zhang PCT was invented by the inventors of the present subject claims:

I understand that the Action asserts that the Zhang disclosures anticipate “a process of preparing adenovirus by preparing a culture of producer cells essentially homogenous with respect to growth, infecting the producer cells with adenovirus between mid-log and stationary phase, and harvesting the adenovirus and placing the virus into a pharmaceutically acceptable composition.” Action at page 9.

Second Zhang Declaration, paragraph 4

This process was invented by me and the other inventors common to the above referenced patent application and the inventors listed on the cited Zhang disclosures.

Second Zhang Declaration, paragraph 5

However, the Examiner, now in possession of the Second Zhang Declaration, nevertheless continues to reject claims 1, 3-9, 13-28, 30, 31, 38-49 and 51-62 as being anticipated by the Zhang PCT because Shawn Gallagher is not listed as an inventor on that reference (Office Action Dated November 17, 2004, page 9). In doing so, the Examiner fails to apply the law of *In re Manger* which demonstrates that when a prior publication is authored by fewer than all the inventors of the present application, that prior publication is not “by another” when the invention described was actually conceived of, researched, and reduced to practice by the inventors of the present application. The Zhang PCT is not proper prior art because the inventive entity of the disclosed but unclaimed subject matter of the reference and the inventive entity of the present application are the same and not “by others” within the meaning of 35 U.S.C. § 102(a). Therefore, Appellants respectfully request that the Board reverse the Examiner’s rejections.

2. The Zhang Patent Is Not Proper Prior Art

Claims 1, 3-9, 13-28, 30, 31, 38-49 and 51-62 are not anticipated by the Zhang Patent (Exhibit 9) reference as further evidenced by Wu under 35 U.S.C. § 102(e) because the Zhang Patent is not proper prior art. The inventive entity of the subject matter disclosed but not claimed in the Zhang Patent is the same inventive entity as the present application and is therefore not “by another” within the meaning of 35 U.S.C. § 102(e).

These claims were rejected in the Office Action Dated June 3, 2004 and maintained in the Office Action Dated November 17, 2004. In the June Office Action, the Examiner states that the Zhang Patent anticipates “a process of preparing adenovirus by preparing a culture of producer cells essentially homogenous with respect to growth, infecting the producer cells with adenovirus between mid-log and stationary phase, and harvesting the adenovirus and placing the virus into a

pharmaceutically acceptable composition.” Action at page 9. The Examiner then notes that the 35 U.S.C. § 102(e) rejection could be overcome by “showing under 37 C.F.R. § 1.132 that any invention disclosed but not claimed in the reference was derived from the inventor of this application and is thus not the invention ‘by another.’” (June 3, 2004 Office Action, page 8, paragraph 2).

In response, the Appellants submitted the Second Zhang Declaration to demonstrate that the invention disclosed but not claimed in the Zhang Patent was invented by the inventors of the present subject claims. As stated above, Dr. Zhang indicates in his declaration that that the disclosed but unclaimed subject matter of the Zhang Patent was invented by him and the other co-inventors of the present application. Therefore, the inventive entity is the same.

However, having reviewed the Second Zhang Declaration, the Examiner continues to focus on only the named inventors of the Zhang patent—those who contributed to the *claimed* invention—instead of focusing the inventive entity of the *unclaimed* subject matter. In doing so, the Examiner maintains the rejection of claims 1, 3-9, 13-28, 30, 31, 38-49 and 51-62 as being anticipated by the Zhang Patent under 35. U.S.C. § 102(e) because Shawn Gallagher is not listed as an inventor on that reference (Office Action Dated November 17, 2004, page 7). As indicated by Dr. Zhang, the unclaimed subject matter in the Zhang Patent was invented by the *same* inventive entity of the present application. This inventive entity includes Shawn Gallagher.

The Examiner indicates that regardless of the fact that the present invention was disclosed but not claimed in the Zhang Patent, the entire reference is available as prior art (Office Action Dated November 17, 2004, page 6). However, if the entire disclosure is available as prior art, then the entire disclosure has two different inventive entities. The claimed subject matter which does not anticipate the present invention is “by another,” and the subject matter which

does so *is not* “by another.” Therefore the Zhang Patent is not proper prior art under 35 U.S.C. § 102(e).

The reliance on the Wu reference as prior art by the Examiner in the Office Action Dated June 3, 2004 and maintained in the Office Action Dated November 17, 2004 is inappropriate at least because Wu is not prior art. Wu incorporates by reference the same application that is now the Zhang Patent. The portion of the Zhang patent that is summarized by Wu is that which was disclosed but not claimed and which was derived from the same inventive entity of the present subject claims. Therefore, reliance on Wu as prior art is inappropriate. Furthermore, to the extent that the Wu reference comments about the teachings of the Zhang Patent, the statements do not evidence what one of ordinary skill in the art understood from the Zhang reference because the inventors on the Wu reference are the inventors of the Zhang reference. Therefore, Appellants respectfully request that the Board reverse the Examiner’s rejections.

3. Dependent Claims Are Not Anticipated by Either Zhang Reference As Further Evidenced by Wu

Claims 3-9, 13-28, 30, 31 and 38-46 ultimately depend from claim 1 and claims 48, 49 and 52-62 ultimately depend from claim 47, and none of these claims are anticipated by either Zhang reference because they are not proper prior art. Therefore, Appellants respectfully request that the board reverse the Examiner’s rejection.

D. Claims 10-12 and 29 Are Not Rendered Obvious by Huyghe as Applied to Claims 1, 3, 8, 9, 13-25, 31, 32, 38, 47, 49 and 51-62 Under 35 U.S.C. § 103(a)

In the Office Action Dated June 3, 2004 and maintained in the Office Action Dated November 17, 2004, the Examiner rejects claims 10-12 and 29 under 35 U.S.C. §103(a) as being unpatentable over Huyghe as applied to claims 1, 3, 8, 9, 13-25, 31, 32, 38, 47, 49, and 51-62. The Examiner contends that although Huyghe does not teach specific cell numbers to be plated (claims 10-12), those numbers would be a subjective determination by one of ordinary skill in

the art based on many factors, such as type of cell, the condition of the cells before plating, and the nature of the cell's division, etc. It concludes that it would be *prima facie* obvious for one skilled in the art to determine the appropriate number of cells to plate for each situation encountered. The Action also alleges that although Huyghe does not teach a harvested adenovirus with the characteristics listed in claim 29, this claim is obvious because it would have been obvious to one of ordinary skill in the art to test any for any of these properties to ensure a good yield of adenovirus.

To establish a *prima facie* case of obviousness, three basic criteria must be met. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art reference (or references when combined) must teach or suggest all the claim limitations. The teaching or suggestion to make the claimed combination and the reasonable expectation of success must both be found in the prior art, and not based on applicant's disclosure.

In re Vaeck, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cir. 1991).

The Examiner has not made a showing of a *prima facie* case of obviousness with respect to claims 10-12. First, Huyghe does not motivate or suggest to a person of ordinary skill in the art that one would determine seeding density in order to infect producer cells with adenovirus between mid-log and stationary phase of growth. Second, the adenovirus production method taught by Huyghe would not provide a person of ordinary skill in the art a reasonable expectation of success in infecting producer cells with adenovirus between mid-log and stationary phase of growth because, as the Examiner states on page 8, paragraph 3 of the Office Action Dated November 17, 2004, "seeding density is crucial for log phase." Yet by the Examiner's own admission, Huyghe does not mention seeding density. Third, instead of teaching the claim limitations of claims 10-12 (infection between mid-log to stationary phase of growth as determined by seeding density), Huyghe teaches infection of producer cells based on percentage

of confluency. Therefore, the Examiner has not made a *prima facie* case of obviousness and Appellants respectfully request that the Board reverse the Examiner's rejections.

Claim 29 is not rendered obvious by Huyghe because Huyghe does not teach or suggest an adenovirus preparation meeting the specific purity limitations set forth in the claim. The Examiner, on page 8, paragraph 5 to page 9 of the Office Action Dated November 17, 2004, states that it would be *prima facie* obvious to one of ordinary skill in the art to "test any one of the properties listed to ensure a good yield of adenovirus." However, claim 29 is not directed to testing adenovirus preparations but to adenovirus preparations that meet these limitations. Huyghe does not teach or suggest adenoviral preparations that meet these limitations. Therefore, the Examiner has not made a *prima facie* case of obviousness and Appellants respectfully request that the Board reverse the Examiner's rejection.

E. Claims 10-12 and 29 Are Not Rendered Obvious by the Zhang PCT As Applied to Claims 1, 3-9, 13-28, 30, 31, 38-49, and 51-62 Under 35 U.S.C. § 103(a)

Claims 10-12 and 29 are not rendered obvious by the Zhang PCT reference because at the very least, the Zhang PCT is not proper prior art.

In the Office Action Dated June 3, 2004 and maintained in the Office Action Dated November 17, 2004, the Examiner contends that although neither Zhang Reference teaches specific cell numbers to be plated (claims 10-12), those numbers would be a subjective determination by one of ordinary skill in the art based on many factors, such as type of cell, the condition of the cells before plating, and the nature of the cell's division, etc. The Examiner concludes that it would be *prima facie* obvious for one skilled in the art to determine the appropriate number of cells to plate for each situation encountered. The Examiner also alleges that although the Zhang References do not teach a harvested adenovirus with the characteristics

listed in claim 29, this claim is obvious because it would have been obvious to one of ordinary skill in the art to test any of these properties to ensure a good yield of adenovirus.

Appellants refer to the previously submitted Second Zhang Declaration. As discussed above, the Zhang References are not proper prior art. Accordingly, Appellants respectfully request that the Board reverse the Examiner's rejections.

F. Claims 10-12 and 29 Are Not Rendered Obvious by the Zhang Patent As Applied to Claims 1, 3-9, 13-28, 30, 31, 38-49, and 51-62 Under 35 U.S.C. § 103(a)

Claims 10-12 and 29 cannot be rendered obvious by the Zhang Patent because either 1) the Zhang Patent is not proper prior art as discussed above, or 2) the Zhang Patent qualifies as prior art under 35 U.S.C. § 102(e), in which case it is not available for a determination of obviousness because both the Zhang Patent and the present invention are under a common obligation of assignment. Therefore, Appellants respectfully request that the Board reverse the Examiner's rejection.

G. Claims 2 and 50 Are Not Rendered Obvious by Huyghe As Applied to Claims 1, 3, 8, 9-12, 13-25, 29, 31, 38, 47, 49, and 51-62 Above and Further in View of Graham and Leu Under 35 U.S.C. § 103(a)

Claims 2 and 50 are not rendered obvious by Huyghe and further in view of Graham and Leu. Even if combinable, a person of ordinary skill in the art would not be motivated to incorporate the methods to produce hepatitis A virus of Leu into the adenovirus production step of Huyghe to prepare adenovirus of the instant invention. Furthermore, there is no expectation of success in using a methodology designed to produce hepatitis A virus in human embryonic lung fibroblasts (MRC-5 cells) in the adenovirus production step of Huyghe, which deals with adenovirus grown in 293 cells.

Claim 2, which is dependent on claim 30, which is dependent on claim 1, is directed towards infecting producer cells with adenovirus between late-log and stationary phase of growth

when infection takes place in a bioreactor system, a microcarrier culture system, a multiplate culture system, a perfused packed bed culture system, or a microencapsulation culture system. Claim 50 is dependent on claim 47 and is directed toward a method of producing adenovirus including culturing the producer cells and infecting the cells with adenovirus between late-log and stationary phase of growth. Neither claim 50 nor claim 47 require the use of a particular culture system.

The Examiner contends in the Office Action Dated June 3, 2004 and maintained in the Office Action Dated November 17, 2004 that although Huyghe does not teach infecting cells at late-log to stationary phase of cell-growth, Leu teaches infecting cells at late-log. In the Office Action Dated November 17, 2004, the Examiner contends that one would have been motivated to propagate the adenovirus of Huyghe with the cell culture method steps of infection of Leu to increase the amount of adenovirus produced in cell culture. The Action also alleges that one of ordinary skill in the art would have had a reasonable expectation of success because Leu teaches that a wide range of adenoviruses may be propagated to generate vaccines using the method steps. The Graham reference is used by the Examiner to support the argument that cells can be infected with 80% to 90% confluency, which, the Examiner argues, demonstrates that the teachings of Leu are applicable to adenovirus infection at late-log phase of growth. Therefore, the Examiner concludes, in the Office Action Dated June 3, 2004 and maintains in the Office Action Dated November 17, 2004, the invention would have been *prima facie* obvious for one skilled in the art absent unexpected results. Appellants respectfully traverse this rejection.

In order to establish a *prima facie* case of obviousness, three basic criteria must be met: 1) there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to

combine the reference teachings; 2) there must be a reasonable expectation of success; and 3) the prior art reference (or references when combined) must teach or suggest all the claim limitations. MPEP § 2142. *See also In re Vaeck*, 947 F.2d 488, 20 USPQ 2d 1438 (Fed. Cir. 1991) (emphasizing that the teaching or suggestion to make the claimed combination and the reasonable expectation of success must be both found in the prior art, and not based on applicant's disclosure).

1. There is no suggestion or motivation to combine Huyghe, Leu, and Graham

One criteria necessary in establishing a *prima facie* case of obviousness is that there be some suggestion or motivation to combine Leu and Graham with the teachings of Huyghe. Appellants contend that there is no such motivation for several reasons.

a) Leu is Replete With References to Hepatitis A Virus

The Examiner contends that one of ordinary skill in the art at the time the invention was made would have been motivated to have propagated the adenovirus encoding p53 of Huyghe with the cell culture method steps of infection taught by the Leu reference. However, in no Office Action does the Examiner explain why a person of ordinary skill in the art would turn to the Leu reference for the cell culture method steps of adenoviral infection. There is no credible reason why a skilled artisan, armed with the teaching of Huyghe would turn to the Leu reference to achieve production levels of adenovirus, as in the claimed invention. Appellants have previously pointed out and to so again, that there is no mention made of adenovirus throughout the entire Leu reference. Rather, Leu is replete with references to hepatitis A virus. Specifically Leu teaches a method of producing large quantities of hepatitis A virus. However, the present invention is specific to adenovirus. \

As would be known by anyone of ordinary skill in the art, hepatitis A virus is structurally and structurally distinct from that of adenoviruses. The distinctions between hepatitis A virus and adenovirus have been previously submitted to the Examiner in a Declaration of Zhuyuan Zhang under 37 C.F.R. § 1.132, Dated March 3, 2004 (Exhibit 5) (Zhang Declaration).

b) Hepatitis A Virus and Adenovirus Have Distinct Properties

The hepatitis A virus, based on its structure and biological properties, is classified as belonging to the viral family *Picornaviridae*. An adenovirus, on the other hand, has a different structure and biological properties and is classified as belonging to the family *Adenoviridae*. These distinctions are supported by the Zhang Declaration and are summarized below.

Adenoviruses contain double-stranded DNA approximately 36 kb in length. They are covered by a capsid 70-100 nm in diameter. This differs from hepatitis A virus in that hepatitis A viruses contain positive single-stranded RNA of approximately 7.2-8.4 kb in length and possess a small 27-32 nm, protein capsid. Summarized from the Zhang Declaration, paragraph 5.

Further, adenovirus replication and assembly occur in the nucleus. After adenoviral replication, the adenovirus is assembled inside the host cell's nucleus resulting in optimal stability for the virus being at a pH between 7.0-8.5. In contrast, hepatitis A viruses undergo replication and assembly in the cytoplasm. In addition, hepatitis A viruses are heat and acid stable, and relatively detergent resistant, such that stability of hepatitis A virus is best achieved at pH 3.0 or lower. These features of hepatitis A virus are not shared by adenovirus, and would strongly suggest that purification methods useful for hepatitis A virus would not necessarily be relevant to adenovirus. Summarized from the Zhang Declaration, dated March 3, 2004, paragraphs 6-7.

The preceding descriptions and comparisons of adenovirus to hepatitis A virus provide substantial evidence that, due to the numerous dissimilarities, a teaching relating one viral type would not necessarily be applicable to the other. Thus, there is no *a priori* expectation that propagation of hepatitis A viruses would provide appropriate means for adenovirus preparations.

Zhang Declaration, paragraph 12.

Due to the failure of the Leu reference to mention adenoviruses and the numerous dissimilarities between hepatitis A virus and adenovirus, such as morphology, genome structure, and lifecycle, there would be no motivation to combine Leu with Huyghe.

2. There Is No Reasonable Expectation of Success in Growing the Adenovirus of Huyghe with the Cell Culture Method Taught by Leu

The second criteria necessary in establishing a *prima facie* case of obviousness is that there be some reasonable expectation of success in growing the adenovirus by combining the teachings of Leu and Graham with the teachings of Huyghe. Appellants contend that there is no such expectation of success for following reasons.

a) The Wide Range of Adenoviruses Mentioned in Leu Do Not Belong to the Family Adenoviridae

In the Office Action Dated November 17, 2004, the Examiner contends that a person of ordinary skill in the art at the time the invention was made would have a reasonable expectation of growing the adenovirus of Huyghe with the cell culture method steps taught by Leu because Leu teaches that a wide range of viruses may be propagated to generate vaccines using the method steps and Graham teaches infecting cells at 80%-90% confluency with adenovirus. Appellants contend however, that none of the eight other viruses mentioned in Leu (column 5, lines 29-31) as cited by the Examiner, belong to the family *Adenoviridae*. In Examples 8 and 9, Leu teaches the propagation of varicella virus and mumps virus respectively.

It is known in the art that both Herpesvirus and Paramyxovirus consist of an envelope with surface projections whereas adenoviruses have no envelope. Thus, these viruses require an

isotonic osmolarity in order to achieve stability and prevent damage to their envelope membrane, whereas adenoviral stability may be achieved at relatively hypertonic osmolarity as it has no envelope. The replication cycle of Herpesvirus and Paramyxovirus is also distinct from that of adenovirus and involves proteins of the respective envelopes. Following entry into the host cell, these viruses require specific enzymes, thymidine kinase and RNA-dependent RNA polymerase respectively, for transcription.

To draw an analogy to the Examiner's reasoning, one would have a reasonable expectation of success culturing human neurons by the same method that one would use to culture mouse lymphocytes because regardless of the genetic and morphological differences, both are eukaryotic cells. Merely believing that Leu is applicable to the general viral propagation art does not make it so, and does not make its teachings relevant to adenoviruses.

For the reasons stated above, the Examiner has failed to demonstrate that a person of ordinary skill in the art would have a reasonable expectation of success in combining the adenovirus of Huyghe with the cell culture method taught by Leu.

3. The Prior Art References of Huyghe, When Combined with Leu and Graham Do Not Teach All the Claim Limitations

The third criteria necessary in establishing a *prima facie* case of obviousness is that the reference adenoviruses produced by Huyghe must be combined with the references of Leu and Graham to teach all the claim limitations. Appellants contend that the combination of these references do not teach the claim limitations.

The Examiner has not pointed out, and the Appellants cannot find, any mention of 293 cells within the entire Leu reference. In further examination of the Leu reference, it would seem that several cell types are mentioned; among these are Vero, CEF, BHK, HFF, MDBK, and MRC-5 (Column 9, lines 28-31). The Leu reference does give examples of infection with two

different viruses in MRC-5 (human embryonic lung fibroblasts), and one virus used to infect CEF cells (chick embryo fibroblasts) (Examples 1-8 and Example 9, respectively). The Appellants are unsure as to how a person of ordinary skill in the art would combine infecting MRC-5 cells with hepatitis A virus with the methods of Huyghe, involving 293 cells and adenovirus, as the Examiner has failed to adequately explain how infection of any cell type mentioned by Leu relates to what Huyghe purportedly teaches.

The Examiner has failed to adequately explain how Leu can be combined with Huyghe to teach any benefit of employing the particular timing of infecting the cell culture at the time that is recited in the claims—that is, late log or early stationary phase of growth. The Examiner has not shown that Leu teaches that this timing leads, for example to increased hepatitis A virus production. Further, the Examiner has not shown whether Leu teaches that using the mid-log phase of growth, late-log phase of growth, or stationary phase of growth to infect cell culture would in any way be beneficial in achieving increased production of any other virus actually mentioned, much less adenovirus.

The Graham reference is used by the Examiner to support the argument that cells can be infected at 80% to 90% confluency, which she argues demonstrates that the teachings of Leu are applicable to adenovirus infection at late-log phase of growth. However, Appellants contend that there would be no motivation to combine the teaching of Leu with the teachings of Huyghe and Graham. The only motivation that the Examiner provides for turning to the Leu reference for its timing of infection is that Leu lists several virus families for which its cell culturing techniques could be used. None of these families include Adenoviridae. Therefore, this rejection is impermissible. “[I]t is impermissible within the framework of 35 U.S.C. § 103 to pick and choose from any one reference only so much of it as will support a given position to the

exclusion of other parts necessary to the full appreciation of what such reference fairly suggests to one skilled in the art.” *Bausch & Lomb, Inc. v. Barnes-Hind/Hydrocurve, Inc.*, 230 U.S.P.Q. 416 (Fed. Cir. 1986). It appears that the Examiner is seeking to employ hindsight reconstruction to pick and choose among isolated disclosures in the prior art to render the instant invention as obvious. *See In re Fritch*, 972 F.2d 1260, 23 U.S.P.Q.2d 1780, 1784 (Fed. Cir. 1992). The Federal Circuit has repeatedly held that such hindsight reconstruction is an improper basis for a §103 rejection. *See id.*

Similarly, there is no teaching or suggestion from Huyghe that there is a problem that needs to be solved. Consequently, neither Leu or Huyghe recognize a problem and thus there is no reason why a skilled artisan would turn to this particular aspect of virus production described in Leu. A “patentable invention may lie in the discovery of the source of a problem even though the remedy may be obvious once the source of the problem is identified.” *In re Sponnoble*, 160 U.S.P.Q. 237, 243 (C.C.P.A. 1969). A corollary to these principles is where the prior art fails to recognize the existence of a problem in the first place. In this regard, the CCPA has held that it is improper to conclude that an invention is obvious absent evidence that one of skill would have recognized that an underlying problem existed. *In re Nomiya*, 184 U.S.P.Q. 607 (CCPA 1975).

As noted in passing above, the caselaw strongly supports a conclusion of non-obviousness in the present case. The Supreme Court, in *Eibel Process*, noted that the discovery of the source of a known problem is strong evidence of non-obviousness:

... we must not lose sight of the fact that one essential part of Eibel’s discovery was that the trouble causing the defective paper product under high machine speed was in the disturbance and ripples some ten feet from the discharge and that they were due to the unequal speeds of stock and wire at that point and could be remedied by equalizing the speeds. The invention was not the mere use of a high or substantial pitch to remedy a known source of trouble. ***It was the discovery of the source not before known and the application of the remedy for which Eibel was entitled to be rewarded in his patent.***

Eibel, 261 U.S. at 67-68. (emphasis supplied). See also *Sponnoble*, 160 U.S.P.Q. at 243 (“It should not be necessary for this court to point out that a patentable invention may lie in the discovery of the source of a problem even though the remedy may be obvious once the source of the problem is identified.”).

Perhaps most relevant to our situation is the *Nomiya* case, where the CCPA, relying on the principles of *Eibel Process* and *Sponnoble*, held that invention is found in the recognition of a previously unknown problem:

If, as appellants claim, there is no evidence of record that a person of ordinary skill in the art at the time of appellants’ invention would have expected the problem in the IGFET to exist at all, ***it is not proper to conclude that the invention which solves this problem, which is claimed as an improvement of the prior art device, would have been obvious to that hypothetical person of ordinary skill in the art.*** This significance of evidence that a problem was known in the prior art is, of course, that knowledge of a problem provides a reason or motivation for workers in the art to apply their skill to its solution. Logically, the instant situation is one step removed from the circumstances illustrated by [*Eibel Process*], where the rippling in paper produced on Fourdrinier paper-making machines was known, but the source of the problem was not.

Nomiya, 184 U.S.P.Q. at 612-13. (emphasis supplied)

In the present case, as in *Nomiya*, the Examiner fails to present substantial evidence that those of skill recognized the existence or source of the problem with the large-scale production of adenovirus.

Thus, given the reasons discussed above and provided in the earlier declaration of Dr. Shuyuan Zhang, one of ordinary skill in the art would not be motivated to combine the teachings of Leu with the teachings of Huyghe (or Zhang *et al.*, discussed below) to prepare an adenovirus of the instant invention. Accordingly, the rejection based on the combination of Leu with that of Huyghe does not meet a necessary criteria required to establish a *prima facie* case of obviousness. Consequently, Appellants respectfully request that the Board reverse the Examiner’s rejection.

H. Claims 2 and 50 Are Not Rendered Obvious by the Zhang References As Applied to Claims 1, 3- 31, 38- 49 and 51-62 Above and Further in View of Graham and Leu Under 35 U.S.C. § 103(a)

The Examiner rejects claims 2 and 50 under 35 U.S.C. §103(a) as being unpatentable over either Zhang reference as applied to claims 1, 3-32, 38-49, and 51-62, and further in view of Graham and Leu, as stated in the June 3 and November 17, 2004 Office Actions. More specifically, the Examiner contends that although neither Zhang reference teaches infecting cells at late-log to stationary phase of cell-growth, Leu teaches infecting cells at late-log. The Examiner argues that one would have been motivated to propagate the adenovirus of Zhang with the cell culture method steps of infection of Leu to increase the amount of adenovirus produced in cell culture. The Examiner also alleges that one of ordinary skill in the art would have had a reasonable expectation of success because Leu teaches that a wide range of viruses may be propagated to generate vaccines using the method steps. The Graham reference is used by the examiner to support the argument that cells can be infected at 80% to 90% confluency, which she argues demonstrates that the teachings of Leu are applicable to adenovirus infection at late-log phase of growth. The Examiner concludes that the invention would have been *prima facie* obvious for one skilled in the art absent unexpected results. Appellants respectfully traverse this rejection.

Given the reasons discussed above and provided in the Zhang Declaration, one of ordinary skill in the art would not be motivated to combine the teachings of Leu with the teachings of either Zhang reference as well to prepare an adenovirus of the instant invention. Accordingly, the rejection based on the combination of Leu with that of Zhang *et al.* does not meet a necessary criteria required to establish a *prima facie* case of obviousness. Consequently, Appellants respectfully request that the Board reverse the Examiner's rejection.

I. Claims 26-28 Are Not Rendered Obvious by Huyghe As Applied to Claims 1, 3, 8, 9-12, 13-25, 29, 31, 38, 47, 49 and 51-62 Above and Further in View of Graham et al. Under 35 U.S.C. § 103(a)

Claims 26-28 are not made obvious by Huyghe further in view of Graham because this combination does not teach the claimed invention. In the Office Action Dated November 17, 2004, the Examiner contends that Huyghe, when combined with Graham *et al.*, teaches the claimed invention of claims 26-28. However, the prior art references must teach or suggest all the claim limitations. *See In re Vaeck*, 947 F.2d 488.

Claims 26-28 depend from claim 1, which, as stated *supra*, is not made obvious by Huyghe as this combination does not teach the claimed invention. The addition of the teachings of Graham *et al.* does not cure the deficiencies of Huyghe. Graham *et al.* is cited only for its teaching of 5% deoxycholate, which does not provide any motivation to combine with the reference of Huyghe.

In light of all the foregoing, Appellants respectfully request that the Board reverse the Examiner's rejections to claims 26-28.

J. Claims 4, 30, 39-46 and 48 Are Not Rendered Obvious by Huyghe As Applied to Claims 1, 3, 8, 9-12, 13-25, 29, 31, 38, 47, 49 and 51-62 Above and Further in View of Garnier and Spier Under 35 U.S.C. § 103(a)

Claims 4, 30, 39-46 and 48 are not rendered obvious by Huyghe in view of Garnier (Exhibit 12) and Spier (Exhibit 13) under 35 U.S.C § 103(a) because there is no suggestion or motivation to combine Garnier or Spier with Huyghe.

The Examiner contends, in both the June and November 2004 Office Actions, that a person of ordinary skill in the art would have been motivated to use the system of Garnier with the method of Huyghe to produce large quantities of adenovirus. Additionally, in the Office Action Dated November 17, 2004, the Examiner asserts that a person of ordinary skill in the art would have been motivated to a culture system described by Spier in the manner and system

Huyghe and Garnier, and that one of ordinary skill in the art would have had a reasonable expectation of success because Garnier uses a bioreactor system and Spier reviews various types of bioreactor systems.

The Examiner has not shown where any of these references, alone or when combined, teaches infection at mid-log phase. As discussed above, Huyghe does not teach infection of cells with adenovirus at mid-log phase. In fact, the February 13, 2002 Declaration of Shawn Gallagher under 37 C.F.R. § 1.132 provides strong evidence supporting the conclusion that the cells were, at best, in early log phase. Neither Garnier nor Spier corrects this defect.

Moreover, there would be no motivation to combine the very different goals of Garnier with the goals of the present application. Garnier concerns only the increased production of heterologous proteins using an adenovirus expression system and does not concern the production of adenovirus. In the Office Action Dated November 17, 2004, the provides no support for her contention that an increase in a heterologous protein necessarily implies an increase in adenovirus production. In fact, the reference makes it clear that viral production is secondary to heterologous protein production as the authors describe how production of the heterologous protein, PTP1C, is *greater* than the production of the adenovirus structural proteins.

Active PTP1C was produced at a constant rate and reached a level of $18\mu\text{g}/10^6$ cells in 3 days post infection. Furthermore, as can be seen by SDS-PAGE of the cell extract (Fig 2.b) for samples at 0, 24, 48, and 72 hpi, the PTP1C band at 68kDa followed the same accumulation kinetics as the activity assay. It can also be seen in Fig.2(b) that PTP1C constituted the most abundant cellular protein, overtaking the hexon and 100k viral proteins.

Garnier, page 150, paragraph 2.

It would seem that the references teaches conditions to increase production of the heterologous proteins at the expense of virus production, thereby teaching away from the

claimed invention. The Federal Circuit held in *In re Mills*, 916 F.2d 680, 682 (Fed. Cir. 1990), that the mere fact that combination or modification of a reference or references is possible does not establish obviousness of the resultant combination unless the prior art also suggests the desirability of the combination, *i.e.*, unless the prior art provides motivation to produce the resultant combination. *Id.*; *see also* MPEP § 2143.01, page 2100-131. In this case, there is no such motivation.

Finally, because Huyghe does not teach infection of cells at mid-log phase of growth to stationary phase of growth, and Garnier teaches conditions to increase the production of heterologous proteins at the expense of virus production, a person of ordinary skill in the art would not be motivated to combine the teachings of Spier with the methods of Garnier and Huyghe to create the invention of the present application. Furthermore, Spier does not appear to even mention adenoviruses or any bioreactor system that might support their propagation.

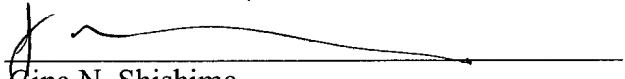
For the reasons stated above, a proper *prima facie* case of obviousness has not been made. Accordingly, Appellants respectfully request that the Board reverse the Examiner's rejection.

VIII. CONCLUSION

For the above-argued reasons, Appellants respectfully request that the Board reverse the Examiner's rejections of the claims. Appellants have provided arguments that overcome the pending rejections. Appellants respectfully submit that the Office Action's conclusion that the claims should be rejected is unwarranted. It is therefore again requested that the Board overturn the Action's rejections.

Please date stamp and return the enclosed postcard to evidence receipt of this document.

Respectfully submitted,


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APPENDIX A

CLAIMS APPENDIX

1. (Previously presented) A process for preparing adenovirus, the process comprising:
 - (a) preparing a culture of producer cells in a selected media;
 - (b) infecting producer cells in the culture with the adenovirus, wherein the producer cells are infected between mid-log phase of growth and stationary phase of growth; and
 - (c) harvesting adenovirus from the cell culture.
2. (Previously presented) The process of claim 30, wherein the producer cells are infected with the adenovirus between late-log phase and stationary phase of growth.
3. (Previously presented) The process of claim 1, wherein the producer cells are essentially homogeneous with respect to the phase of cell growth.
4. (Original) The process of claim 1, wherein the producer cells are perfused for at least a portion of the time that the cells are cultured.
5. (Original) The process of claim 4, wherein the producer cells are perfused at a rate that will maintain a glucose level of between about 0.5 and about 3.0 gm glucose/liter.
6. (Original) The process of claim 5, wherein the producer cells are perfused at a rate that will maintain a glucose level of between about 0.7 and about 2.0 gm glucose/liter.
7. (Original) The process of claim 6, wherein the producer cells are perfused at a rate that maintains a glucose level of between about 1 and about 1.5 gm glucose/liter.
8. (Original) The process of claim 1, wherein the producer cells are seeded into the culture medium and allowed to attach to a culture surface for between about 3 hours and about 24 hours prior to infection with adenovirus.
9. (Original) The process of claim 1, wherein the culture medium is at least partially recirculated during the adenovirus infection step.

10. (Original) The process of claim 1, wherein the culture medium is seeded with between about 0.5×10^4 and about 3×10^4 cells/cm².
11. (Original) The process of claim 10, wherein the culture medium is seeded with between about 7.5×10^3 and about 2.0×10^4 cell/cm².
12. (Original) The process of claim 11, wherein the culture medium is seeded with between about 9×10^3 and 1.5×10^4 cells/cm².
13. (Original) The process of claim 1, wherein the harvested adenovirus is subjected to purification and placed into a pharmaceutically acceptable composition.
14. (Original) The process of claim 13, the adenovirus is purified by steps which include chromatography.
15. (Original) The process of claim 14, wherein the chromatography step involves subjecting the adenovirus to more than one chromatographic separations.
16. (Original) The process of claim 14, wherein the chromatography step involves subjecting the adenovirus to only one chromatographic separation.
17. (Original) The process of claim 16, wherein the chromatographic separation includes ion-exchange chromatography.
18. (Previously presented) The process of claim 1, wherein said adenovirus is a replication-deficient adenovirus encoding a selected gene operably linked to a promoter.
19. (Original) The process of claim 18, wherein said replication deficient adenovirus is lacking at least a portion of the E1 region.
20. (Original) The process of claim 19, wherein said producer cells complement the growth of replication deficient adenovirus.
21. (Original) The process of claim 1, wherein said producer cells are selected from the group consisting of 293, PER.C6, 911 and IT293SF cells.

22. (Original) The process of claim 21, wherein said producer cells are 293 cells.
23. (Original) The process of claim 18, wherein said selected gene is selected from the group consisting of antisense *ras*, antisense *myc*, antisense *raf*, antisense *erb*, antisense *src*, antisense *fms*, antisense *jun*, antisense *trk*, antisense *ret*, antisense *gsp*, antisense *hst*, antisense *bcl* antisense *abl*, Rb, CFTR, p16, p21, p27, p57, p73, C-CAM, APC, CTS-1, *zac1*, scFV *ras*, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, MMAC1, FCC, MCC, BRCA2, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, GM-CSF G-CSF, *mda-7*, thymidine kinase or p53.
24. (Original) The process of claim 23, wherein said selected gene is a p53 gene.
25. (Original) The process of claim 18, wherein said promoter is an SV40 IE, RSV LTR, β -actin, CMV-IE, adenovirus major late, polyoma F9-1, or tyrosinase promoter.
26. (Original) The process of claim 1, wherein the adenovirus is harvested by steps that include lysing the producer cells by means other than freeze-thaw.
27. (Original) The process of claim 26, wherein the producer cells are lysed by means of a detergent lysis.
28. (Original) The process of claim 26, wherein the producer cells are lysed by means of autolysis.
29. (Previously presented) The process of claim 1, further comprising purifying the harvested adenovirus to obtain a purified adenovirus composition having one or more of the following properties:
- (a) a virus titer of between about 1×10^9 and about 1×10^{13} pfu/ml;
 - (b) a virus particle concentration between about 1×10^{10} and about 2×10^{13} particles/ml;
 - (c) a particle:pfu ratio between about 10 and about 60;
 - (d) having less than 50 ng BSA per 1×10^{12} viral particles;
 - (e) between about 50 pg and 1 ng of contaminating human DNA per 1×10^{12} viral particles,

- (f) a single HPLC elution peak consisting essentially of 97 to 99% of the area under the peak.
30. (Previously presented) The process of claim 1, wherein infecting producer cells in the culture with the adenovirus occurs in a bioreactor system, a microcarrier culture system, a multiplate culture system, a perfused packed bed reactor system, or a microencapsulation culture system.
31. (Previously presented) The process of claim 29, further comprising formulating the purified adenovirus composition into a pharmaceutically acceptable composition.
32. (cancelled)
33. (Previously presented) The process of claim 31, wherein the pharmaceutically acceptable composition is administered to a subject.
34. (Previously presented) The process of claim 33, wherein the subject is a mammal.
35. (Previously presented) The process of claim 34, wherein the mammal is a human or a mouse.
36. (Previously presented) The process of claim 33, wherein administering is intravenously, intradermally, intramuscularly, intraarterially, intralesionally, percutaneously, subcutaneously, or by inhalation.
37. (Previously presented) The process of claim 36, wherein administering is intratumorally.
38. (Previously presented) The process of claim 1, wherein the adenovirus is a recombinant adenovirus.
39. (Previously presented) The process of claim 1, wherein the producer cells are cultured in a bioreactor system.
40. (Previously presented) The process of claim 39, wherein the bioreactor system is a stirred tank reactor.

41. (Previously presented) The process of claim 39, wherein the bioreactor system is a airlift reactor.
42. (Previously presented) The process of claim 39, wherein the bioreactor system is a sparged bioreactor.
43. (Previously presented) The process of claim 1, wherein the producer cells are cultured in a microcarrier culture system.
44. (Previously presented) The process of claim 1, wherein the producer cells are cultured in a multiplate cell culture system.
45. (Previously presented) The process of claim 1, wherein the producer cells are cultured in a perfused packed bed reactor system.
46. (Previously presented) The process of claim 1, wherein the producer cells are cultured in a microencapsulation culture system.
47. (Previously presented) In a method for producing adenovirus that includes culturing producer cells and infecting the cultured producer cells with an adenovirus, wherein the improvement comprises infecting said producer cells with the adenovirus when the cells in culture are between mid-log phase of growth and stationary phase of growth.
48. (currently amended) A method of claim 47, wherein the further improvement comprises infecting the cultured producer cells in a bioreactor system, a microcarrier culture system, a multiplate culture system, a perfused packed bed reactor system, or a microencapsulation culture system.
49. (Previously presented) A method of claim 47, wherein the improvement further comprises harvesting adenovirus from the cell culture.
50. (Previously presented) A method of claim 47, wherein the improvement further comprises infecting producer cells in a culture with adenovirus between late-log phase of growth and stationary phase of growth.

51. (Previously presented) A method of claim 47, wherein said adenovirus is a recombinant adenovirus.
52. (Previously presented) A method of claim 51, wherein said recombinant adenovirus comprises a selected gene operably linked to a promoter.
53. (Previously presented) A method of claim 47, wherein said adenovirus is a replication-deficient adenovirus.
54. (Previously presented) A method of claim 53, wherein said replication deficient adenovirus is lacking at least a portion of the E1 region.
55. (Previously presented) A method of claim 47, wherein said producer cells complement the growth of replication deficient adenovirus.
56. (Previously presented) A method of claim 55, wherein said producer cells are selected from the group consisting of 293, PER.C6, 911 and IT293SF cells.
57. (Previously presented) A method of claim 56, wherein said producer cells are 293 cells.
58. (Previously presented) A method of claim 47, wherein the producer cells are essentially homogeneous with respect to the phase of cell growth.
59. (Previously presented) A method of claim 52, wherein said selected gene is selected from the group consisting of antisense *ras*, antisense *myc*, antisense *raf*, antisense *erb*, antisense *src*, antisense *fms*, antisense *jun*, antisense *trk*, antisense *ret*, antisense *gsp*, antisense *hst*, antisense *bcl* antisense *abl*, Rb, CFTR, p16, p21, p27, p57, p73, C-CAM, APC, CTS-1, zac1, scFV *ras*, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, MMAC1, FCC, MCC, BRCA2, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, GM-CSF G-CSF, mda-7, thymidine kinase or p53.
60. (Previously presented) A method of claim 59, wherein said selected gene is a p53 gene.

61. (Previously presented) A method of claim 52, wherein said promoter is an SV40 IE, RSV LTR, β -actin, CMV-IE, adenovirus major late, polyoma F9-1, or tyrosinase promoter.
62. (Previously presented) A method of claim 49, wherein the improvement further comprises purifying the harvested adenovirus to obtain a purified adenovirus composition having one or more of the following properties:
- (a) a virus titer of between about 1×10^9 and about 1×10^{13} pfu/ml;
 - (b) a virus particle concentration between about 1×10^{10} and about 2×10^{13} particles/ml;
 - (c) a particle:pfu ratio between about 10 and about 60;
 - (d) having less than 50 ng BSA per 1×10^{12} viral particles;
 - (e) between about 50 pg and 1 ng of contaminating human DNA per 1×10^{12} viral particles,
 - (f) a single HPLC elution peak consisting essentially of 97 to 99% of the area under the peak.

APPENDIX B

EVIDENCE APPENDIX

1. Huyghe *et al.*, *Human Gene Therapy* 6:1403-1416 (1995): Cited in Office Action Dated November 17, 2004
2. Declaration of Shawn Gallagher Under 37 C.F.R. § 1.312; Filed February 13, 2002
3. Mediatech Technical Information Bulletin: Cited in Office Action Dated November 17, 2004
4. Robert J. Kuchler, *Biochemical Methods in Cell Culture and Virology*. Stroudsburg, Penn: Dowden, Hutchinsonson & Ross, Inc., 90, 91, 99, 100, 1977: Cited in Office Action Dated November 17, 2004
5. Declaration of Shuyuan Zhang Under 37 C.F.R. § 1.312; Filed March 3, 2004
6. Zhang *et al.* (WO 98/22588): Cited in Office Action Dated November 17, 2004
7. Wu *et al.* (U.S. Patent 6,689,600 B1): Cited in Office Action Dated November 17, 2004
8. Second Declaration of Shuyuan Zhang Under 37 C.F.R. § 1.312; Filed September 3, 2004
9. Zhang *et al.* (U.S. Patent 6,194,191): Cited in Office Action Dated November 17, 2004
10. Graham and Prevec, *In: Methods in Molecular Biology: Gene Transfer and Expression Protocols* 7. Murray, E.J. Editors. Clifton, NJ: Humana Press, 109-128 and 205-225, 1991: Cited in Office Action Dated November 17, 2004
11. Leu *et al.* (U.S. Patent 6,194,210 B1): Cited in Office Action Dated November 17, 2004
12. Garnier *et al.*, *Cytotechnol.*, 15:145-155, 1994: Cited in Office Action Dated November 17, 2004
13. Spier, R.E. and J.B. Griffiths, eds., *Animal Cell Biotechnology*, Vol. 3 (1988): Cited in Office Action Dated November 17, 2004
14. Office Action dated September 13, 2001
15. Office Action dated June 3, 2004
16. Office Action dated November 17, 2004

Purification of a Type 5 Recombinant Adenovirus Encoding Human p53 by Column Chromatography

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ABSTRACT

We have investigated the use of column chromatography for the purification of ACN53, a recombinant adenovirus type 5 encoding the human p53 tumor suppressor protein. Anion exchange, size exclusion, hydrophobic interaction, and metal chelating resins were tested; each was found to have distinct advantages and disadvantages. Based on these data, a rapid method was devised for the purification of ACN53. The resultant product was characterized and compared to cesium chloride density-gradient purified virus by SDS-PAGE, Western blot analysis, absorbance spectrum, total particle-to-infectious particle ratio, expression of p53 gene product in Saos-2 cells, growth inhibition of Saos-2 cells, and contamination by ATCC-293 host cell proteins. The results show that column chromatography offers an alternative to ultracentrifugation for the purification of recombinant adenoviruses for use in human gene therapy trials and other research applications.

OVERVIEW SUMMARY

We have devised a chromatographic protocol for the purification of recombinant adenoviruses intended for use in human gene therapies that allows for production on an industrial scale. This method is intended to replace the current methodology of density-gradient ultracentrifugation. A comparison of the purity and potency of a recombinant adenovirus type 5 bearing the human p53 gene (ACN53) derived from chromatographic and ultracentrifugation methods is presented, and the advantages of virus purification by column chromatography are discussed.

INTRODUCTION

ADVANCES IN GENE THERAPY technology have brought new treatments for cancer and other serious disease states to the stage of clinical trials. In most cases, gene therapy involves viral vectors delivering genes to target cells. In striking contrast to the rapid advances that have taken place in genetic technology, purification of viruses has continued to rely upon density gradient centrifugation as a primary mode of purification for more than 30 years (Green and Pina, 1964). New methodologies for production and purification of viruses are needed if these promising experiments are to result in therapeutic prod-

ucts. The results reported here show that column chromatography offers significant potential as a method for large-scale virus production.

Our research has focused on new therapies based upon tumor suppressor genes and gene products, the two best characterized of which are p53 and retinoblastoma (Rb). Mutations in the p53 gene and subsequent loss of functional p53 protein have been implicated in the malignant behavior of a variety of human tumors (Bartek *et al.*, 1991; Hollstein *et al.*, 1991). Our strategy has been to suppress proliferating cells by introducing the normal p53 gene by infection with a recombinant adenovirus (rAd). The vector chosen for these studies, ACN53, was derived from an adenovirus type 5 (Ad 5) virus that has had the E1 coding sequences replaced with a 1.4-kb full-length p53 cDNA with expression driven by the human cytomegalovirus promoter (Wills *et al.*, 1994). Recombinant virions were produced in the human embryonal kidney cell line 293.

Previous published attempts to purify virus by chromatographic means has focused on size-exclusion chromatography (Hewish and Shukla, 1983; Albrechtsen and Heide, 1990). Size exclusion appears promising for bovine papilloma virus (Hjorth and Moreno-Lopez, 1982), and has been shown to be a superior method for the purification of tick-borne encephalitis virus (Crooks *et al.*, 1990). The use of size-exclusion chromatography has not yet become widespread, but is currently being employed for large-scale production of recombinant retrovirus

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(Mento, S. J., Viagene, Inc. as reported at the 1994 Williamsburg Bioprocessing Conference). Affinity chromatography, mostly using monoclonal antibodies (mAb), has been reported to be an effective method for the purification of antigens of viral origin (Njayou and Quash, 1991). Infective soybean mosaic virus (a virus that can survive pH 3) can be recovered using mAb affinity chromatography (Diacio *et al.*, 1986). Fowler *et al.* (1985) used affinity chromatography and density gradient centrifugation to purify Epstein-Barr virus.

Adenoviruses are large (diameter of approximately 80 nm) and somewhat fragile. A large literature base dealing with the relationship of structure to function has accumulated (for reviews, see Philipson, 1983; Horwitz, 1990). Little has been reported in the literature about chromatographic purification of infectious adenoviruses. Haruna *et al.* (1961) reported encouraging results using DEAE ion-exchange chromatography of Ad 1, 3, and 8. Klemperer and Pereira (1959) and Philipson (1960) reported disappointing results for the use of a similar method with Ad 2 and 5.

Chromatographic resins and equipment have been improved substantially since 1960. Given these recent advances and a wealth of available information about adenoviruses, we decided to revisit ion-exchange chromatography and to examine other modern chromatographic techniques for the publication of adenoviruses.

MATERIALS AND METHODS

Production of infected ATCC 293 cells

293 cells (American Type Culture Collection, CRL 1573) were grown in a 6,000-cm² Cell Factory (Nunc) in a humidified air/7% CO₂ incubator in 1.5 liters of DME high-glucose medium (Irvine Scientific) supplemented with 10% fetal bovine serum (FBS) (Hyclone).

Two to 2.5 days after seeding the Cell Factory, when cell monolayers reached about 50–60% confluency, the cells were infected at a multiplicity of infection (moi) of 5–10 infectious units (IU) per cell in 500 ml of fresh medium. The virus was added to the medium, mixed thoroughly, and introduced to the cells in the unit. Three to 4 days post-infection, the infected cells were ready for harvesting.

Harvest and lysis

When cell monolayers showed signs of detachment from the surface of the Cell Factory, the cells were harvested by gentle tapping and centrifuged in a Beckman TJ-6 at 1,500 rpm for 5 min. Cells were resuspended and pooled in 25 ml of 100 mM Tris buffer pH 8.0 for use in the preparation of ultracentrifuge-derived standard virus. Samples destined for use in chromatography were resuspended in 25 ml of 50 mM HEPES buffer pH 7.5/150 mM NaCl, 2 mM MgCl₂, and 2% sucrose. The cells were lysed at this point by three cycles of freeze-thaw. Following the third cycle, cellular debris was removed by centrifugation in a Beckman TJ-6 at 1,500 rpm for 5 min. The supernatant from this step was adjusted to 2 mM MgCl₂, 2% (wt/vol) sucrose and 2.5% (wt/vol) β -cyclodextrin. Benzonase (American International Chemical, Inc. Natick, MA) was added to a final concentration of 100 units/ml and allowed to

incubate for 1 hr at room temperature. The treated material was clarified by centrifugation in a Beckman TJ-6 at 3,000 rpm for 10 min and filtration through a Gelman Sciences Acrodisc 0.8/0.2- μ m filter.

Preparation of ACN53 standard material (CsCl-ACN53)

Standard recombinant ACN53 virus was prepared by a three-step centrifugation procedure as described (Laver *et al.*, 1971) with the following modifications. Infected cells were lysed by three cycles of freeze-thaw and centrifuged at 15,000 rpm for 10 min, 4°C in a Sorvall SS34 rotor. The pellet was discarded, and the supernatant was treated with Benzonase at 133 U/ml for 30 min at room temperature. The treated material was layered onto a 1.25 g/ml and 1.40 g/ml CsCl discontinuous step gradient in 10 mM Tris pH 8.1, and centrifuged at 30,000 rpm for 60 min, 10°C in a Sorvall TST 41-14 rotor. The virus band from each tube was collected, pooled, mixed with 1.35 g/ml CsCl (in 10 mM Tris pH 8.1), and centrifuged overnight at 45,000 rpm, 10°C in a Beckman VTi 65 rotor. The virus band from each tube was collected and recentrifuged at 45,000 rpm as before for an additional 4 hr. The final virus pool from this step was dialyzed extensively against phosphate-buffered saline (PBS) supplemented with 2% sucrose and 2 mM MgCl₂.

Chromatographic parameters

Column resins were tested for their separation characteristics in 6.6 \times 50-mm (1.7 ml) borosilicate Omnifit columns fitted with 25- μ m polyethylene frits. The columns were mounted on a PerSeptive Biosystems Biocad chromatography workstation. The chromatography was monitored on-line for pH, conductivity, and dual wavelength optical density detection at 280 nm (*A*₂₈₀) and 260 nm (*A*₂₆₀).

Anion-exchange resins were equilibrated in 50 mM HEPES pH 7.5, 300 mM NaCl, 2 mM MgCl₂, 2% sucrose at 1 ml/min. A 50 mM Tris buffer pH 8.0, (with 300 mM NaCl, 2 mM MgCl₂, 2% sucrose) was also used in certain trials for comparison. After samples were loaded and washed to baseline as monitored by absorbance, elution was performed with a 20-column volume 300–600 mM linear NaCl gradient and collected in 0.5-ml fractions. The column was then cleaned with two column volumes of 0.5 M NaOH followed by one column volume of 1.5 M NaCl.

Size-exclusion chromatography experiments were done in a 6.6 \times 500-mm borosilicate Omnifit column packed with Toyopearl HW-75F resin and equilibrated at 1 ml/min in PBS/2% sucrose/2 mM MgCl₂. Injection volumes varied from 50 to 200 μ l depending on the nature of the sample. Fractions of 0.5 ml were collected during elution.

For hydrophobic interaction chromatography, a 50 mM Tris pH 8/(NH₄)₂SO₄ buffer system and toyopearl butyl 650M resin were used. Samples were prepared for loading onto the column by mixing with an equal volume of 3 M (NH₄)₂SO₄ in 50 mM Tris buffer pH 8.0, incubated for 5–10 min, and centrifuged at 3,000 rpm for 5 min in an Eppendorf Microfuge (model 5415c) to remove any precipitate. The column was eluted with a gradient of 1.5 M to 0 M ammonium sulfate over 10 column volumes at 1 ml/min.

The immobilized zinc affinity chromatography (IZAC) system was prepared for metal charging by washing the column

sequentially with one volume of 100 mM EDTA and one volume of 0.2 M NaOH, flushing with water after each step. The matrix was subsequently charged with zinc by injecting one column volume of 100 mM ZnCl₂ in H₂O acidified with 0.5 μ l/ml glacial acetic acid and thoroughly washed in water prior to equilibration in 50 mM HEPES pH 7.5, 450 mM NaCl, 2% sucrose, and 2 mM MgCl₂. Sample loading did not require any prior manipulation; DEAE pool fractions or CsCl-derived material could be injected directly onto the column. After loading, the column was washed with a 10-column volume linear gradient from 50 mM HEPES pH 7.5, 450 mM NaCl, 2% sucrose, and 2 mM MgCl₂ to 50 mM HEPES pH 7.5, 150 mM NaCl, 2% sucrose, 2 mM MgCl₂. Elution was performed with a linear (0–500 mM) glycine gradient in 150 mM NaCl applied over 10 column volumes.

SDS-PAGE analysis

For Coomassie blue staining, 100–200 μ l of a sample (at approximately 1×10^{11} particles/ml) were collected, desalted by trichloroacetic acid precipitation or by dialysis followed by concentration in a Speed-Vac. The sample was then resuspended in SDS-PAGE reducing buffer [125 mM Tris-HCl pH 6.8, 20% glycerol, 4% (wt/vol) SDS, 0.005% bromphenol blue, 0.5% β -mercaptoethanol] to approximately 30 μ l, boiled for 5 min, and loaded onto a 1-mm \times 10-well Novex 8–16% gradient Tris-Glycine minigel. Samples were electrophoresed for 1.5 hr at 140 V. The gel was then fixed in 40% methanol/10% acetic acid for 30 min, and Coomassie stained with the Pro-Blue staining system (Integrated Separation Systems, Natick MA.) according to the vendor's procedure.

Gels that were to be silver stained were loaded with 5–15 μ l of sample. The sample was boiled with an equal volume of reducing buffer and electrophoresed as described for Coomassie detection. Gels were fixed in 10% trichloroacetic acid for 1 hr, washed three times in ultrapure water, and stained with the Daiichi silver staining kit according to the instructions provided (Integrated Separation Systems).

Western blot analysis

An SDS-PAGE gel was run as described with approximately the same loading as that of a silver-stained gel. The bands were then transferred to a PVDF membrane pre-wetted in 100% methanol and equilibrated in Tris-buffered saline (TBS). The gel was also equilibrated in TBS. The proteins were transferred to the membrane using a Bio-Rad semidry transfer apparatus at 25 V for 30 min. The membrane was then blocked in 1% casein/0.01% sodium azide overnight at 4°C or at room temperature for 1 hr, and washed three times with TBS. The membrane was incubated with the primary polyclonal antibody (Cytimmune rabbit IgG α -adenovirus type 5, Lee Biotechnology Research: San Diego, CA) at 5 μ g/ml (in TBS) for 1 hr at room temperature. Following primary incubation, the membrane was washed three times with TBS and incubated with the secondary antibody (Amersham Life Sciences Horseradish peroxidase-conjugated anti-rabbit Ig) diluted to 1 μ l stock antibody/1 ml TBS for 1 hr at room temperature. A final three-time wash was performed with TBS and the membrane incubated with Amersham ECL detection reagents for 1 min, exposed in the dark to Hyperfilm-ECL (Amersham) for various times (sev-

eral seconds to minutes to give a selection of various contrasts), and developed in an X-ray film developer.

Particle number by absorbance at 260 nm in the presence of SDS

The number of total virus particles (total = infection + non-infective particles) can be derived spectrophotometrically for pure virus. For this measurement, the virus sample ($\sim 1 \times 10^{12}$ particles/ml) was diluted 1:10⁴ in 0.1% SDS in PBS. The sample was vortexed for 1 min and then centrifuged at 14,000 rpm in an Eppendorf Microfuge to remove any precipitate. A matched pair of cuvettes were blanked with 0.1% SDS in PBS buffer by running a baseline scan on Shimadzu UV160U spectrophotometer. The SDS-treated virus sample was placed in the sample cuvette and scanned from 220 to 340 nm. If the absorbance between 310 and 320 nm was greater than 0.02, the sample was diluted further and remeasured. The A_{260}/A_{280} ratio was also determined from this scan, and had to be between 1.2–1.3 to ensure that the product was pure enough to calculate particle number. If this condition was met, the absorbance value at 260 nm only was used to calculate the number of virions per ml. The conversion factor of 1.1×10^{12} particles per absorbance unit at 260 nm (Maizel *et al.*, 1968) was used to calculate particle number with approximately 20% error.

Particle number by anion-exchange HPLC

A 1-ml Resource Q (Pharmacia) anion-exchange column was also used to quantitate the number of total viral particles in various samples to $\pm 5\%$. Unlike the measurement of absorbance at 260 nm, this assay may be applied to samples at any state of purification, from crude lysates to final product. Preparation of lysate samples was performed by first centrifuging the sample at $1,300 \times g$ in a microfuge for 5 min. The supernatant was then adjusted to 2 mM MgCl₂ and 100 U/ml of Benzonase was added for 20–30 min at room temperature. The enzymatic treatment was necessary for assaying crude lysates only. Semipure or pure virus did not require such treatment and could be injected directly.

The column was equilibrated in 300 mM NaCl, 50 mM HEPES pH 7.5 at a flow rate of 1 ml/min on a Waters 625 chromatography system equipped with 717plus autosampler and a model 991 photodiode array detector (PDA). The chromatography was monitored on the PDA detector scanning from 210 to 300 nm. Elution was performed with a 2-ml wash in the equilibration buffer followed by a linear 10 ml 300–600 mM NaCl gradient. The resulting virus peak was quantitated at 260 nm by comparison to standard curves constructed by injecting 1×10^8 to 1×10^{11} CsCl purified ACN53 virions that had been characterized for total particles by A_{260} in 0.1% SDS ($\sim 1 \times 10^{12}$ particles/ml).

The assay was independent of injected sample up to a volume of 1 ml or more. Some standard autosamplers are limited to single-injection volumes of not more than 200 μ l; this limitation could be overcome by repeated injections. Washing the column between 200 μ l injections did not affect the chromatography. After the sample loading, the column was washed with two column volumes of equilibration buffer followed by a linear gradient from 300 to 600 mM NaCl in 50 mM HEPES pH 7.5 over 10 column volumes. The gradient was followed

with a two-column volume wash with 600 mM NaCl in 50 mM HEPES pH 7.5. After each chromatographic run, the column was cleaned with 2.5 ml of 1.5 M NaCl in 50 mM HEPES pH 7.5, and then reequilibrated for the next injection. The column was cleaned more vigorously after injection of crude samples by injecting 0.25 to one column volume of 0.5 N NaOH followed by a wash with 1.5 M salt. Injecting NaOH and then running the gradient was a convenient way to accomplish cleaning.

The validity of this assay was demonstrated by several tests. First, the peak area was found to increase linearly with virus particle number when CsCl-ACN53 was applied to the column. The area was also found to increase in the expected manner when uninfected lysates or infected lysates were spiked with increasing amounts of purified virus. Particle numbers measured by anion-exchange HPLC were in agreement with values obtained by spectrophotometry. Anion exchange was the preferred method for measurement of virus particles because of its speed, sensitivity, and accuracy. A full description of this assay will be presented elsewhere (Shabram *et al.*, manuscript in preparation).

Measurement of infectious particles by TCID₅₀ assay

The quantitation of infectious particles was accomplished by an end point titer assay (tissue culture infective dose of 50%, abbreviated TCID₅₀) similar to assays available in the literature (Philipson, 1961). Reagents, a materials list, and instructions for antibody staining are available from Chemicon International, Inc. (cat. # 3130, Adenovirus Direct Immunofluorescence Assay, Temecula, CA).

In brief, 293 cells were plated into a 96-well microtiter plate: 100 μ l of 5×10^5 cells/ml for each well in complete MEM (10% bovine calf serum; 1% glutamine) media (GIBCO BRL). In a separate plate, a 250- μ l aliquot of virus sample diluted 1:10⁶ was added to the first column and was serially diluted two-fold across the plate. Seven rows were used for samples, one was used for a negative control. A 100- μ l aliquot of each well was transferred to its identical position in the ATCC-293 seeded plate and allowed to incubate at 37°C in a humidified air/7% CO₂ incubator for 2 days. The media was then decanted by inversion and the cells fixed with 50% acetone/50% methanol. After washing with PBS, the fixed cells were incubated for 45 min with a FITC-labeled anti-Ad5 antibody (Chemicon International #5016) prepared according to the kit instructions. After washing with PBS, the plate was examined under a fluorescent microscope (490 nm excitation, 520 nm emission) and scored for the presence of label. The titer was determined using the Titerprint Analysis program (Lynn, 1992). Typically, titers for purified material range from 1×10^9 to 2×10^{10} (IU)/ml. The assay can measure infectious titers from 10⁶ to 10¹¹ IU/ml. Error in the assay ranges from 20 to 35%.

Expression of p53 protein

The activity of virus preparations was also tested by assaying for expression of the p53 gene product in Saos-2 cells (ATCC), a p53-negative osteosarcoma cell line. Saos-2 cells were seeded into a 6-well tissue culture plate at a concentration of 5×10^5 cells/well in 3 ml of media: Kaighn's nutrient mixture F12 (GIBCO BRL), DME high glucose (1:1 mixture),

supplemented with 2 mM L-glutamine and 10% fetal bovine serum (HyClone). The cells were incubated in humidified air/7% CO₂ chamber at 37°C for 16–24 hr. Spent media was removed and replaced with 1 ml of fresh media, and the cells were infected at an moi of 20, 40, or 60 using purified virus. After incubation for 1 hr, an additional 2 ml of media was added and allowed to incubate for 8 hr. The cells were then washed once with Dulbecco's-PBS (GIBCO BRL) and lysed by adding 250 μ l of: 50 mM Tris, 0.5% Nonidet P40, 250 mM NaCl, 5 mM EDTA, 5 mM NaF, 5 μ g/ml Leupeptin, and 5 μ g/ml Aprotinin/2 mM PMSF (Boehringer Mannheim). The plate was incubated on ice for 5 min, after which the lysates were transferred to individual 1.7-ml microcentrifuge tubes. These were centrifuged for 45 sec at 14,000 rpm in a microfuge. The supernatants were assayed for the presence of p53 protein by Western blot analysis with the primary anti-p53 monoclonal antibody 1801 (Vector Laboratories, Burlingame, CA) and a 1:1 mixture of sheep anti-mouse IgG-HRP and streptavidin-HRP (Amersham). The p53 protein band was detected using Amersham's ECL detection kit in accordance with the manufacturer's instructions.

Potency assay

The antiproliferative activity of replication-incompetent adenovirus samples expressing p53 protein was assessed using a thymidine incorporation bioassay. Saos-2 cells were seeded in 96-well flat-bottomed microtiter dishes in 100 μ l of Kaighn's nutrient mixture F12:DME high glucose (1/1) supplemented with 2 mM L-glutamine and 10% fetal bovine serum at a density of 1×10^4 per well. After an overnight incubation at 37°C in humidified air/7% CO₂ chamber, ACN53 and AC β GAL (a recombinant adenovirus that expresses β -galactosidase) were added in 100- μ l aliquots; a serial three-fold dilution starting from an moi of 50 was used to infect the cells. Positive and negative control samples were incubated on each microtiter plate. All plates were returned to the incubator for an additional 48 hr. All dilutions were tested in duplicate. Cells were labeled with [³H]thymidine (Amersham) at 0.5 μ Ci/well for 6 hr at 37°C in a 7% CO₂ incubator. Next, cells were detached from the plastic substratum by adding 100 μ l per well trypsin-EDTA (GIBCO BRL) at 37°C for 10 min and transferred to a 96-well glass fiber filter cassette (Packard Instrument Co., Meriden, CT) using a 96-well harvester (Packard Instruments). Sample filter cassettes were wetted with 50 μ l/well MicroScint 20 scintillation cocktail, covered with Top-Seal sealant (Packard Instruments), and loaded into a Packard Top Count scintillation counter for analysis. Samples were counted for [³H] for 1 min. Data (in cpm) were compared with media control values and plotted. Dose-response curves and values for ED₅₀ were determined using the general form of the four parameter logistic equation.

RESULTS

Lysate treatment

Nuclease-treated lysate was clarified to remove cellular debris and undissolved β -cyclodextrin through a combination of centrifugation and filtration techniques. Using analytical anion-

exchange analysis to measure recovery, it was found that centrifugation followed by filtration through a Gelman Sciences Acrodisc 0.8/0.2 μm two-stage syringe filter gave the best recovery (94%). Recovery of ACN53 depended on the pore size and type of membrane used for filtration; passage through 0.45- μm polysulfone, PVDF, and cellulose acetate-based membranes recovered between 34 and 75%.

Figure 1 shows that virus particles sediment during centrifugation of lysate samples. A 5-min centrifugation at $15,000 \times g$ in an Eppendorf Microfuge (model 5415c) at 4°C resulted in a 45% loss of virus particles as measured by anion-exchange HPLC, compared to a centrifugation of an identical sample at $1300 \times g$. The loss of particles in the sample correlates directly with the force of the spin.

Infected cell lysate contains contaminants both host cell and viral in origin. Some of these contaminants could be removed by treatment with nuclease prior to chromatography. Specifically, host cell, non-encapsulated, or incomplete ACN53 nucleic acids could be enzymatically degraded at this stage of the process with the addition of nuclease (Benzonase). Such treatment improved yields in ion-exchange chromatography. Benzonase was removed by subsequent purification steps as assayed by a commercially available ELISA kit (American International Chemical).

β -Cyclodextrin was added to lysate to reduce the contaminant load on a DEAE anion-exchange column (data not shown), providing better purification on the DEAE column and increasing the number of cycles the column could be run.

Anion-exchange chromatography

CsCl-ACN53 was injected onto a Fractogel DEAE-650M column equilibrated in 50 mM Tris pH 8 at 2 ml/min (350 cm/hr) and eluted with a 10-min (11.7 column volume) 0–1.5 M linear NaCl gradient. A single peak was detected with an on-line A_{260}/A_{280} ratio of 1.23. The protein bands present in this fraction reacted with Ad-5 polyclonal antibody upon slot-blot analysis.

Several peaks were resolved when an infected cell lysate sample was applied to the DEAE column (Fig. 2). The composition of the peaks could be deduced from the on-line A_{260}/A_{280} absorbance ratio (as opposed to a separate measurement in a stand-alone spectrophotometer with 0.1% SDS treatment). For example, the peak with a retention time of 9 min has an on-line A_{260}/A_{280} ratio of 0.5, and was mainly protein. The 27-min peak had an on-line A_{260}/A_{280} ratio of 2, suggesting that this material was nucleic acid. The ACN53 virus peak eluted at 19 min with a ratio of 1.23. The identities of these peaks were confirmed by spiking experiments and by running SDS gels of each peak. In our chromatography experiments, an on-line A_{260}/A_{280} ratio of 1.23 ± 0.8 was found to be characteristic of virus peaks.

To assess the purification capabilities of DEAE chromatography, experiments were performed in which both noninfected 293 cell lysate and CsCl-ACN53 were applied to the column (Fig. 3). Most of the host cell material either passed through the column during the load or eluted at an earlier retention time

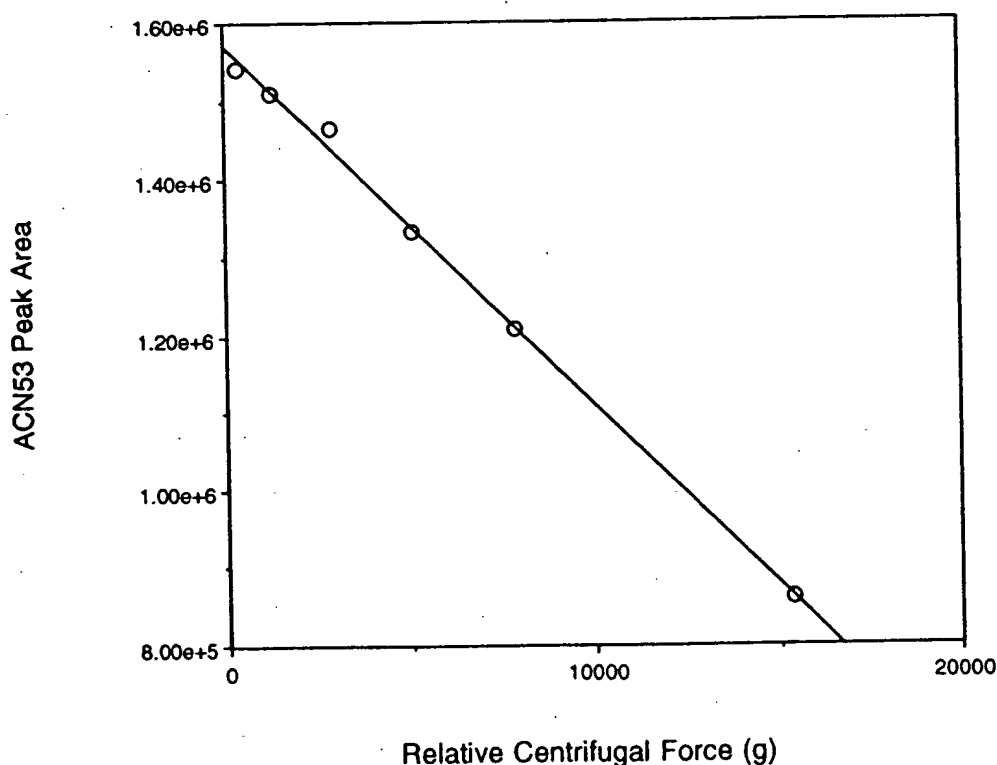


FIG. 1. Recovery of ACN53 from the supernatant of centrifuged lysate. Crude infected cell lysate was centrifuged for 5 min at different speeds in an Eppendorf Microfuge model 5415c at 4°C and analyzed for ACN53 by analytical anion exchange.

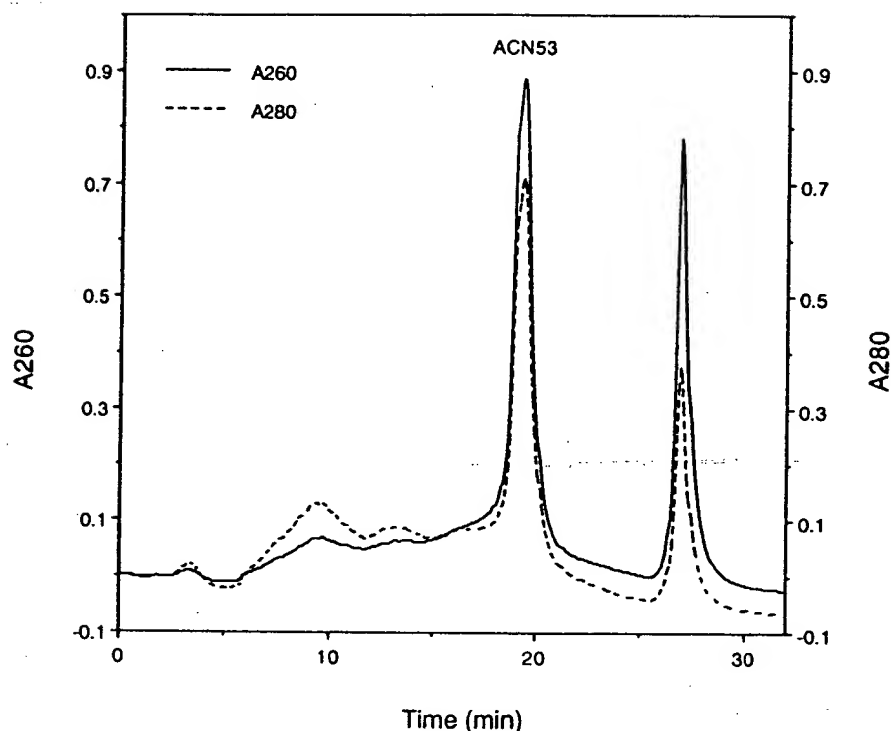


FIG. 2. Identification of ACN53 by dual-wavelength UV absorbance. Infected cell lysate components separated by DEAE chromatography can be identified according to their A_{260}/A_{280} -nm absorbance ratio.

than that of ACN53; however, a small peak eluted with the same retention time as ACN53. From these data it appeared that non-viral contamination of the ACN53 peak might be expected from host cell material. The peak eluting between 22–26 min in the cell lysate sample in Fig. 3 revealed an A_{260}/A_{280} nm ratio of approximately 2, suggesting a high nucleic acid content. This peak was reduced or eliminated by treatment with Benzonase prior to chromatography.

A SDS-PAGE gel of DEAE pooled fractions shows a pattern similar to a CsCl-ACN53 standard (Fig. 4). The absorbance ratio A_{260}/A_{280} of the DEAE pool measured in 0.1% SDS was 1.15–1.20, suggesting contamination by proteins. Product recovery from the column was assessed as 60–80% by infectious titer and analytical anion-exchange HPLC analysis. Most of the recovered infectivity was in the product peak, although a small amount of infectivity was found in the DNA peak and flowthrough pool (1–5%).

Size-exclusion chromatography

The resin chosen for this study was Toyopearl HW-75F because of its large exclusion limit (5×10^6 daltons) a 6.6×500 -mm column was packed and equilibrated at 1 ml/min in PBS, 2% sucrose, and 2 mM $MgCl_2$. A total of 50 μ l of CsCl-ACN53 standard was injected to test the elution characteristics of ACN53 in this system. The resultant peak was very broad, eluting in a fraction size approximately 70% of the total column volume. This effect could not be overcome by increasing the salt concentration to 500 mM NaCl, ruling out ionic interaction of ACN53 with the resin matrix. The recovery off the column was also very low, with only 15–20% of the amount injected being eluted.

Hydrophobic interaction chromatography (HIC)

Two hydrophobic resins, Toyopearl butyl 650M and Toyopearl phenyl 650M, were evaluated. Precipitation experiments performed with CsCl-ACN53 showed that the virus remained soluble in 1.5 M ammonium sulfate, and would therefore remain soluble under the loading conditions. CsCl-ACN53 was injected onto the column, and eluted with a gradient of 1.5 M to 0 M ammonium sulfate over 10 column volumes at 1 ml/min. Both phenyl and butyl resins bound and eluted ACN53, but the butyl column yielded a sharper peak.

After studying the chromatography of cell lysate on butyl 650M, it was determined that DEAE anion exchange was more effective as a first purification step. HIC was then investigated as a second purification step. A DEAE pool derived from an infected 293 cell lysate was diluted 1:1 with 3 M ammonium sulfate and purified over a butyl-HIC column. No precipitate was observed during this step. In the resultant chromatogram, the virus peak was well resolved from other components. Recovery of ACN53 ranged from 5 to 30% as measured by both infectious titer and analytical anion-exchange methods. After further analysis of HIC chromatography of the DEAE-derived virus pool indicated there was a problem with degradation of virus during HIC treatment, we decided to examine metal affinity chromatography.

Immobilized zinc affinity chromatography (IZAC)

The interaction of virions with metals has been inferred from studies of viruses and bacteriophages (Lark and Adams, 1953, Brakke, 1956). We investigated the metal-binding properties of ACN53 by testing its ability to adsorb to a metal affinity col-

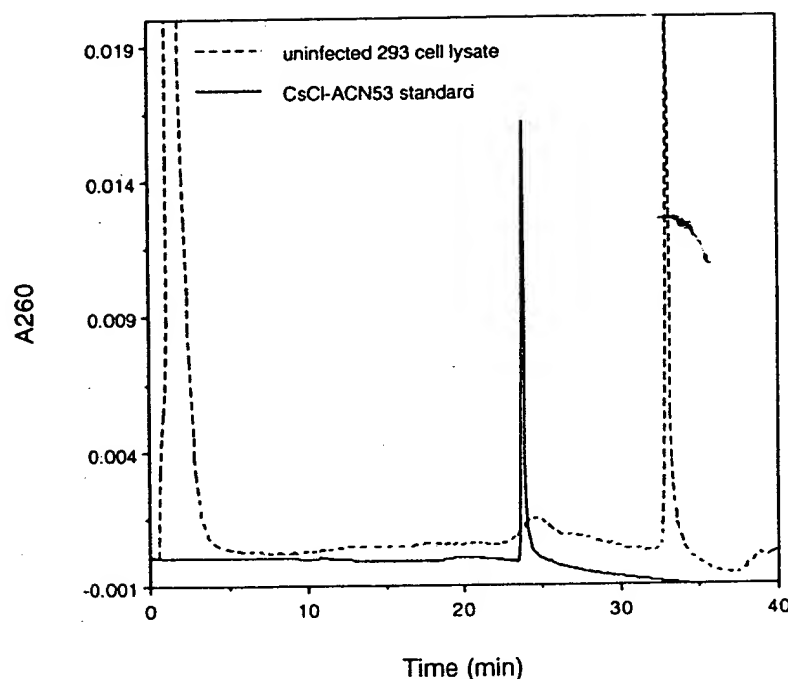


FIG. 3. Comparison of CsCl-ACN53 and host-cell contaminant retention times during DEAE purification. An uninfected 293 host-cell lysate blank was chromatographed over a DEAE column to assess how much contamination would coelute with the virus. The elution of CsCl-ACN53 is overlaid to compare retention times.

umn loaded with zinc ions. An ACN53-DEAE fraction pool purified over IZAC is shown in Fig. 5. Analysis of an IZAC fraction pool gives a yield of 49–65% and an A_{260}/A_{280} ratios of 1.22–1.25. A gel and Western blot comparison of CsCl-ACN53, DEAE-purified, and DEAE-IZAC-purified material can be seen in Figs. 4 and 6. The CsCl-ACN53 and DEAE/IZAC materials were very similar, and the DEAE-only purified material was less pure by these criteria. The interaction of ACN53 with this column was shown to be zinc specific; injection of CsCl-ACN53 onto an uncharged column (a column not preloaded with zinc) resulted in a shift of the virus peak to the flowthrough.

Experiments were designed to study the effect of different IZAC buffer and elution systems. When a DEAE-ACN53 pool was split in half and purified over IZAC in HEPES pH 7.5 and Tris pH 8 buffer systems, the HEPES buffer yielded 2.5-fold more viral particles as measured by anion-exchange HPLC. IZAC could be run in the presence of 2% sucrose and 2 mM $MgCl_2$ without affecting the chromatography. Experience derived from CsCl-ACN53-based material indicated that sucrose and magnesium may help stabilize the virus, especially when freezing and thawing final product (data not shown).

The use of copper as the metal ion and imidazole as the elution agent were also tested (for a general review of metal affinity chromatography, see Kato *et al.*, 1986; Belew *et al.*, 1987). Of the four systems, zinc/glycine, zinc/imidazole, copper/glycine, and copper/imidazole, the zinc/glycine system performed best in terms of virus recovery. Decreasing pH gradients can also be used for elution but were avoided in the case of ACN53 because of its sensitivity to low pH.

ACN53 seems sensitive to abrupt changes in salt concentration. This phenomenon was first seen in HIC chromatography, and was also detected in the investigation of IZAC. When a DEAE fraction pool was diluted from its ionic strength of ap-

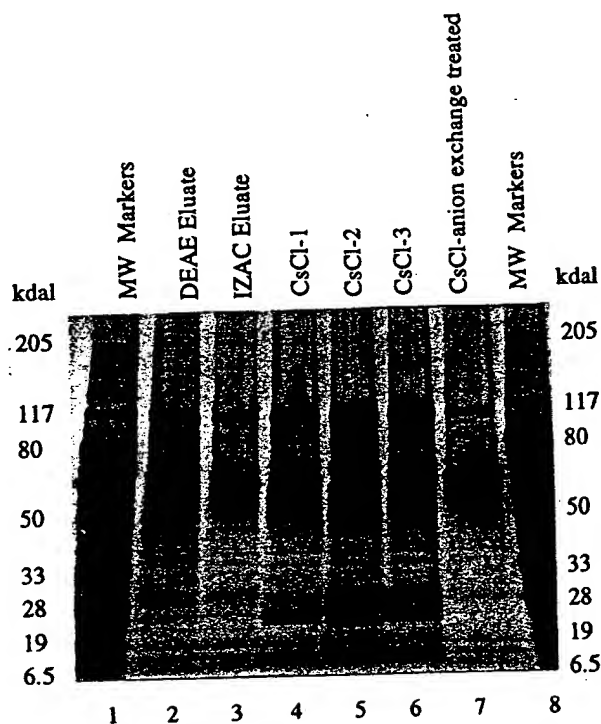


FIG. 4. SDS-PAGE comparison of ACN53 derived from column chromatography and CsCl ultracentrifugation. Samples were electrophoresed on an 8–16% gradient gel and silver stained. Lanes 2 and 3 are DEAE and IZAC eluate pools, respectively. Lanes 4–6 represent three different preparations (CsCl-1, CsCl-2, CsCl-3) of CsCl-ACN53 run side by side. Lane 7 represents the ACN53 peak recovered when a sample of CsCl-ACN53 is purified over an analytical anion-exchange HPLC column. Lanes 1 and 8 contain standard molecular weight markers.

proximately 450 mM to 150 mM NaCl and loaded onto an IZAC column equilibrated at 150 mM NaCl, a flowthrough peak with a high $A_{260/280}$ was observed. When the loading conditions were revised such that the IZAC column was equilibrated in 50 mM HEPES pH 7.5, 2% sucrose, 2 mM $MgCl_2$, and 450 mM NaCl (approximating the ionic strength conditions of the DEAE fraction pool), the size of the flowthrough peak was diminished. The objective was to load the DEAE eluate onto the IZAC column iso-osmotically, and gradually decrease the salt concentration to 150 mM in a controlled fashion to avoid virus degradation due to osmotic shock. This was accomplished with a 10-column volume linear 450–150 mM NaCl gradient. ACN53 was subsequently eluted with the same glycine gradient used before. Iso-osmotic loading and gradual desalting increased the product yield by 12% over a column that had been equilibrated and loaded at 150 mM NaCl.

Comparison of ACN53 virus purified by CsCl method and DEAE-IZAC chromatography

Virus preparation purified by ultracentrifugation and by column chromatography were compared by six criteria: SDS-PAGE, Western blots, A_{260}/A_{280} ratio in SDS, the ratio of total virus particles to infectious virus particles, expression of p53 gene product, growth suppression by the gene product, and an immunoassay for the presence of host-cell proteins in the final product.

Figure 4 shows a silver-stained SDS polyacrylamide gel analysis of several purified ACN53 fractions. At least 20 bands

can be discerned in each of the three lots purified in CsCl; the relative intensities of the bands show some variation from lot to lot, particularly in the low-molecular-weight (10–35 kilodaltons) region. Virus purified by DEAE and IZAC shows a simpler band pattern, with less protein visible in the low-molecular-weight region. Although not shown in this figure, silver-stained gels of other lots purified by DEAE-IZAC also showed consistent band patterns similar to that seen in Fig. 4, lane 3. One of the lots purified using CsCl (Fig. 4, lane 6) was subjected to further purification by anion-exchange chromatography (see lane 7). Low-molecular-weight proteins were removed by this procedure, resulting in a band pattern very similar to virus purified by the DEAE-IZAC method.

Western blots developed with anti-Ad 5 polyclonal antibody revealed several bands. The differences between banding patterns of ACN53 in various states of purification can be seen in Fig. 6. The major difference can be seen in the relative intensities of the bands in the 10- to 35-kilodalton regions between the CsCl-ACN53 preparations and the chromatographic pools. This data sheds little light upon the differences in patterns seen in silver-stained SDS gels. It is possible that loosely bound virus proteins are removed by chromatography; alternatively, the low-molecular-weight proteins absent, or present in reduced levels, in DEAE-IZAC purified material may be cellular contaminants removed by chromatography but not by CsCl density-gradient centrifugation. In either case, the reduced levels of these proteins did not change the infectivity of the virus.

The absorbance ratio (A_{260}/A_{280}) is important because it is related to the ratio of protein and DNA in the product (discussed

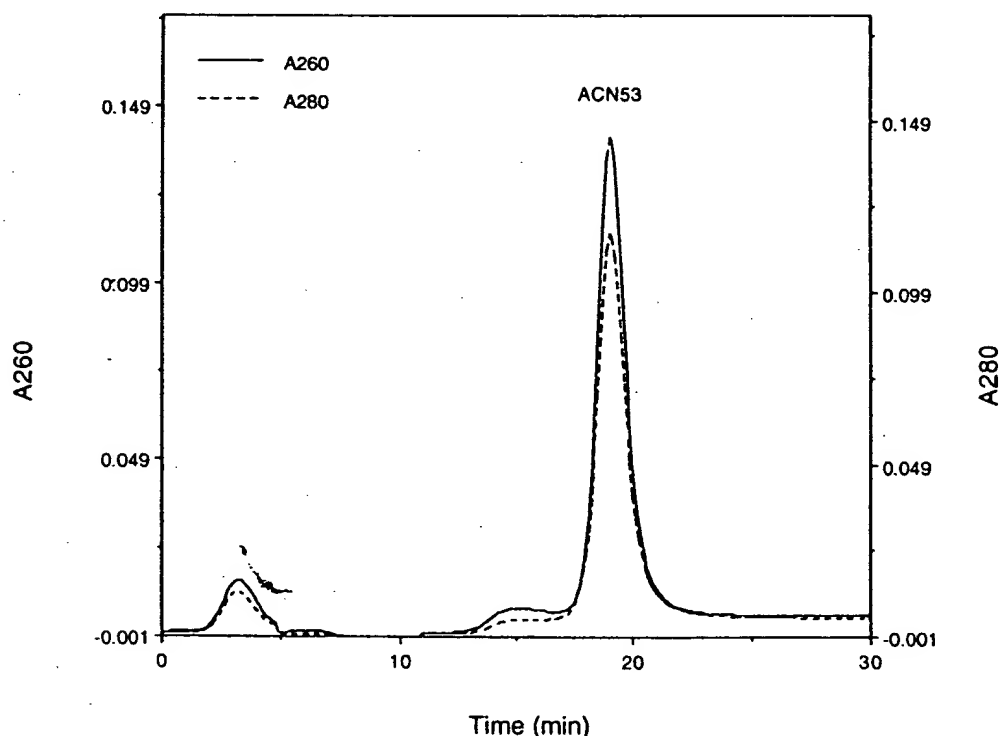


FIG. 5. Immobilized metal affinity chromatography of a DEAE-ACN53 fraction pool. A DEAE-purified ACN53 fraction pool was injected onto a 6.6×50 -mm TosoHaas AF chelate 650M column charged with $ZnCl_2$ and eluted with a linear 0–500 mM glycine gradient.

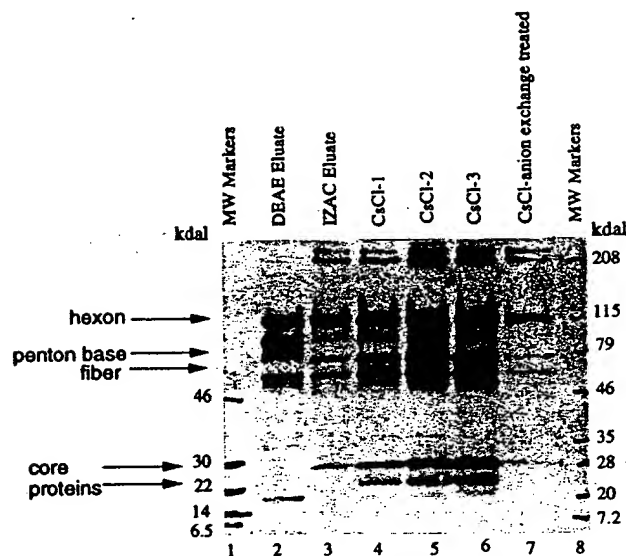


FIG. 6. Western blot comparison of ACN53 derived from column chromatography and CsCl ultracentrifugation. Samples identical to those described in Fig. 4 were electrophoresed on an 8–16% gradient gel and transferred to a PVDF membrane. The blot was incubated first with 5 μ g/ml Cytimmune rabbit IgG anti-Ad 5 antibody, then with Amersham's horseradish peroxidase-conjugated anti-rabbit Ig (NA934) and developed using electrochemical detection.

below), and can be used as a measure of product purity. For various CsCl-ACN53 virus preparations measured in 0.1% SDS, the ratio was found to range from 1.2 to 1.3. The variation in the measured ratio reflects lot-to-lot variability as well as error in measurements. DEAE-IZAC products yielded ratios of 1.22–1.25, which are within the acceptable range. The A_{260}/A_{280} ratio seemed less variable for DEAE-IZAC preparations than for CsCl-ACN53 virus preparations.

When samples purified by DEAE chromatography were subjected to this analysis, the range of ratio values for DEAE fraction pools was lower (1.14–1.20). These values could be related to how the fractions were pooled: wider pools gave lower numbers, consistent with the presence of contaminating proteins. The second chromatography step (IZAC) removed these proteins and improved the A_{260}/A_{280} to values typical of CsCl-purified material (Table 1).

The ratio of total virus particles to infectious viral particles can vary widely from preparation to preparation. Values for CsCl-derived viruses ranged from $20(\pm 7):1$ to $130(\pm 45):1$. The calculation of this ratio in crude lysates or semipure fractions has been made possible by the anion-exchange particle assay. It cannot be performed by A_{260} measurements as described in the Materials and Methods section. For crude samples, interfering absorbance from contaminants is too high. It is possible through the use of analytical anion exchange to observe if and how this ratio changes during purification, *i.e.*, whether the final product's total virus particle-to-infectious virus particle ratio is determined by the original ratio present in the raw material, or if it can be improved during purification. In following this value during a chromatographic purification, the total virus particle-to-infectious virus particle ratio of the crude lysate was

$60(\pm 21):1$ (Table 2). DEAE-purified ACN53 had a particle ratio of $82(\pm 29):1$, while DEAE-IZAC, buffer-exchanged final product had a particle ratio of $88(\pm 31):1$.

The overall yield of total virus was approximately 32%; that of infectious virus was approximately 22%. These values are comparable to recovery values determined for ultracentrifugation procedures we currently use for the preparation of CsCl-ACN53. The yields from ultracentrifugation purifications was measured by analysis of crude lysate and final product by anion-exchange HPLC and infectious titer.

The recombinant adenovirus was designed to transfer a gene to a cell lacking a functional P53 gene. To assay for this activity, we incubated P53 null Saos-2 cells with DEAE and DEAE-IZAC virions, and after allowing for sufficient infection, assayed the cells for production of the p53 gene product by Western-blot analysis. The results are shown in Fig. 7. p53 gene expression can be seen in the semipure DEAE-ACN53 as well as the DEAE-IZAC-ACN53-treated cells. The lower levels of expression in lanes 5 and 6 were due to the fact that they were run at a lower moi than the DEAE samples.

The ability of the gene product to suppress the growth of Saos-2 cells was assayed by measuring [3 H]thymidine incorporation (Fig. 8). Three different viral constructs were assayed for growth inhibition effects: CsCl-ACN53, DEAE-IZAC-ACN53, and CsCl-AC β GAL. Both of the p53 gene-bearing adenoviruses were able to inhibit the incorporation of [3 H]thymidine, while the CsCl-AC β GAL virus had no effect.

We assayed several types of samples for the presence of host cell contamination by Western blot analysis using polyclonal antibodies raised against 293 cell components. The results indicated that the final products of CsCl or DEAE-IZAC purification contained no detectable host cell contaminants. In the case of a partially purified DEAE-ACN53 fraction, there was a small amount of contamination seen: three major bands and several minor ones. The majority of host cell contamination was recovered in the flowthrough portion of the DEAE step. Contaminants that copurified with ACN53 in the DEAE step were removed by zinc affinity chromatography, and were recovered in IZAC flowthrough fractions prior to the introduction of the glycine gradient.

TABLE 1. YIELD AND PURITY OF CHROMATOGRAPHICALLY PRODUCED ACN53 BASED ON TOTAL PARTICLES

Step	Total viral particles ^a	Yield	A_{260}/A_{280} ratio ^b	Purity ^c
Lysate	3×10^{12}	—	—	3%
DEAE load	3×10^{12}	—	—	—
DEAE eluate	2×10^{12}	67%	1.17 ± 0.03	92%
IZAC load	1.52×10^{12}	—	—	—
IZAC eluate ^d	7.14×10^{11}	47%	1.23 ± 0.02	98%
Overall	—	32%	—	—

^aDetermined by HPLC analytical anion-exchange assay.

^bMeasured in 0.1% SDS.

^cMeasured by integration of HPLC chromatogram at 260 nm.

^dAssayed after dialysis into the storage buffer.

TABLE 2. YIELD AND PURITY OF CHROMATOGRAPHICALLY PRODUCED ACN53 BASED ON INFECTIVE PARTICLES

Step	IU/ml ^a	Total IU	Yield	Specific activity (P/IU) ^b
Lysate	1×10^{10}	5.0×10^{10}	—	60(±21):1
DEAE load	1×10^{10}	5.0×10^{10}	—	—
DEAE eluate	4.87×10^9	2.44×10^{10}	49%	82(±29):1
IZAC load	4.87×10^9	1.85×10^{10}	—	—
IZAC eluate ^c	2.7×10^9	8.1×10^9	44%	88(±31):1
Overall	—	—	22%	—

^aDetermined by TCID₅₀.^bThe ratio of noninfectious particles (P) to infectious particles (infectious units, IU) as determined by analytical anion exchange and TCID₅₀ assays.^cAssayed after dialysis into the storage buffer.

DISCUSSION

Detection of ACN53 in process samples

There are several methods available for the detection of viral product in process samples. These vary according to specificity, sensitivity, ease of use, throughput, and versatility in terms of identity and quantitation. TCID₅₀ is very sensitive (10^5 – 10^{11} IU/ml) and has the advantage of being able to identify infective viral particles. It is not the assay of choice, however, for in-process sample analysis because of the time required (3 days). It also has the greatest error (35%). Absorbance measurements performed in a spectrophotometer can be used quickly for quantitation of total particles ($\pm 10\%$), but can only be used to measure the concentration of pure virus.

The quickest and most convenient assay is the analytical anion-exchange assay. This is capable of measuring total virus particles in any sample, from crude lysate to pure material in less than 30 min. It is by this method that we have been able to quantitate stepwise recoveries ($\pm 5\%$) during our purification process. With this assay, the identity, purity, and quantity of the virus can be established simultaneously by the UV absorbance characteristics.

The use of a dual-wavelength on-line spectrophotometric detector monitoring absorption at 260 (DNA λ^{\max}) nm and 280 (protein λ^{\max}) nm can provide real-time identification of ACN53 particles. This is possible because the virus is composed of a complex of DNA and protein in a unique ratio as compared to free proteins (unassembled viral or free host-cell) or uncomplexed nucleic acids. A spectrum of pure DNA reveals an A_{260}/A_{280} ratio of 1.8–2, whereas pure protein has a ratio of 0.4–0.6. A mixture of the DNA and protein has an A_{260}/A_{280} ratio somewhere in-between depending on the relative amounts of each constituent. A spectrum of CsCl-purified, infective ACN53 has a distinct A_{260}/A_{280} ratio of 1.2–1.3. A dual-wavelength chromatogram or on-line photo-diode array detector can easily distinguish between fractions containing mostly DNA, protein or virus (Fig. 2). Measurements of this sort can also be carried out on purified material in a benchtop spectrophotometer in the presence of 0.1% SDS for greater precision. Values <1.2 indicate contaminating protein, whereas values >1.3 indicate excess exogenous DNA present in the product.

SDS-PAGE and Western blot analysis are standard methods

for the detection of proteins and are able to handle a large number of samples in a time frame of hours. The difficulties encountered here are concentration of dilute samples and interference by high salt. Furthermore, because ACN53 is a mixture of proteins, DNA, and carbohydrates, stained gels do not reveal a distinct single band but rather a complex pattern that must be compared to a CsCl standard. Coomassie blue or silver staining may be used. SDS-PAGE analysis is more easily interpreted as the sample becomes more pure, but is difficult to apply in crude lysates.

Western blot analysis is more specific than a stained gel and can unambiguously identify the presence of viral proteins in a complex mixture. This technique also reveals a pattern rather than a single band. Some column fractions will have unassembled viral proteins that are Western positive but are considered

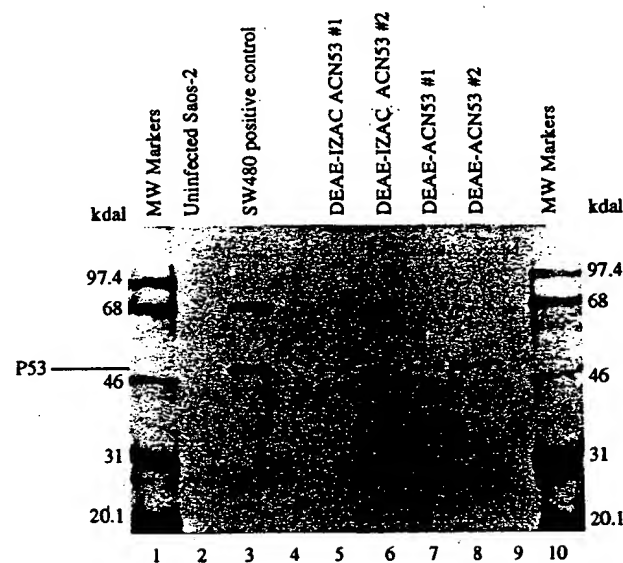


FIG. 7. Expression of p53 gene product in Saos-2 cells. Two different lots of chromatographically produced ACN53 were assayed by Western blot for their ability to affect gene transfer to p53-null Saos-2 cells. The semipure DEAE fractions are shown in lanes 7 and 8, the final product in 5 and 6. p53-expressing SW480 cells were used as a positive control; uninfected Saos-2 cells were used as a negative control.

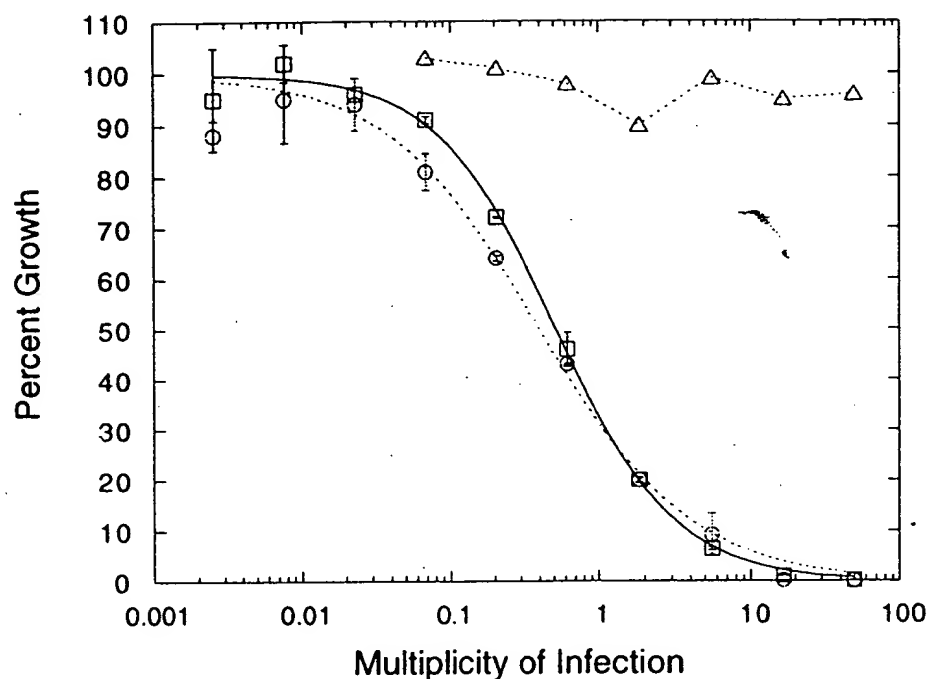


FIG. 8. Growth Inhibition of Saos-2 cells by ACN53. [^3H]Thymidine incorporation was measured in Saos-2 cells treated with DEAE-IZAC-ACN53 (squares) CsCl-ACN53 (circles), and AC β GAL (triangles). The percent inhibition of growth was calculated relative to a control sample in which cells were treated with media only. Calculated ED_{50} values for the samples were: DEAE-IZAC-ACN53, 0.56 ± 0.04 moi; CsCl-ACN53, 0.45 ± 0.05 moi.

as impurities because they represent incomplete or degraded virus particles. For example, previrions form a lower-density band in the CsCl ultracentrifugation of ACN53. Electron micrographs show previrions to be comprised of incomplete viral particles (Moncany *et al.*, 1980). It is difficult to distinguish these inactive previrions from infective virions by Western blot or SDS-PAGE alone; additional information must be acquired (biological assays) and used in conjunction with gels and blots.

Chromatography of ACN53

The treatment of lysate with nuclease is important for two reasons. First, it is necessary to remove unwanted DNA from the product, and second, to improve the performance of the chromatography in terms of yield and purity. Our goal was to obtain and characterize a nuclease that would be available in large quantity for a commercial process. We have chosen Benzonase for these reasons.

Clarification of the treated lysate was accomplished by a combination of filtration and centrifugation. Centrifugation was used to remove cellular debris, but needed to be brief to minimize loss of product (Fig. 1). The particles are large enough to be prone to sedimentation by centrifugation at low speed, as demonstrated in this study, as well as by sedimentation in ultracentrifuges. The yield as a function of centrifugal force can be quickly measured by analytical anion exchange. Loss of product during clarification by filtration was similarly evaluated and optimized for our vector. The types of filters examined in this study were 25-mm syringe tip units; as greater quantities of raw material become available, study on larger filters will be feasible.

The power of the analytical anion-exchange assay is evident in its speed, versatility, and throughput. We now have a fast method for the detection of total viral particles in cell culture samples, as well as process step samples. Having an assay of this sort is critical for quantitating step yields during purification and for protocol optimization, and can be applied many ways, from the optimization of growth conditions in culture to the stability of viral particles under a variety of parameters. The assay was derived from data collected in the study of DEAE chromatography of CsCl-purified virus. The adaptations required to change from a preparative mode to an analytical one were to select a higher-resolution column that could withstand multiple injections of crude lysate, and its installation on a high-performance chromatographic system. Qualitatively, the chromatograms obtained from a preparative DEAE column and an analytical quarternary amine are similar. The two anion-exchange methods differ in that one has been optimized for production and the other for quantitation.

Overall, the characteristics of DEAE chromatography were found to be very consistent, and loading studies with high titer lysate (3×10^{12} virus particles/ml) showed a linear response between volume injected and ACN53 peak area recovered (data not shown). Elution of a DEAE column by introduction of a linear salt gradient gave three major peaks. The first of these was a protein peak with a A_{260}/A_{280} ratio of ~ 0.5 . Next was the ACN53 peak ($A_{260}/A_{280} = 1.23$) followed by a DNA ($A_{260}/A_{280} = 2$) peak at the end of the gradient. This holds true whether run in a HEPES or a Tris buffer system. If run at pH 7.5, less contaminating material bound to the column; pretreatment of lysate with β -cyclodextrin removed additional contaminants. Further studies on DEAE chromatography buffer sys-

tems showed that 2% sucrose and 2 mM MgCl_2 (which have been implicated in other studies in our laboratory for optimal activity; data not shown) did not interfere with the chromatography. Chromatography in phosphate buffers gave poor yields and less well-resolved peaks (consistent with Philipson, 1960). HEPES pH 7.5, NaCl, sucrose, and MgCl_2 was selected as the buffer system. DEAE chromatography yielded a high degree of initial purification. Immunochemical analysis of a DEAE fraction pools using polyclonal antibodies directed against ATCC-293 cell components revealed contaminating host cell proteins present. IZAC purification of the DEAE pool removed these contaminants.

The behavior of ACN53 on a size-exclusion column was explored with the goal of taking advantage of the large size of virus particles. The molecular weight of a virus particle can be estimated to be approximately 2×10^8 daltons with a diameter of 70–90 nm (Green *et al.*, 1967; Van der Eb *et al.*, 1969). The expectation was that this technique could separate mature virions from proteins and DNA fragments. Unassembled or immature virions should elute differently because of their less compact architecture. Most of the literature available concerning the use of chromatography for the purification of virus focuses on size-exclusion techniques (Hjorth and Moreno-Lopez, 1982; Hewish and Shukla, 1983; Olivon *et al.*, 1986; Albrechtson and Heide, 1990; Crooks *et al.*, 1990). We found several disadvantages involved with the use of size-exclusion chromatography for the purification of virions. Most commercially available resins are intended for separation of proteins of less than 10^6 daltons or for desalting/buffer exchange applications. It may be possible to utilize size exclusion by collecting ACN53 in the excluded frontal peak and the lower-molecular-weight contaminants in later fractions. Another concern is the injection volume constraint of a sizing column and the need to concentrate virus prior to injection onto the column. The use of a size-exclusion column increased the process time by introducing the need for sample concentration steps prior to injection and after product collection.

The positive attributes of butyl-HIC chromatography were that it could purify a DEAE fraction pool to an A_{260}/A_{280} nm ratio of 1.2–1.25 without any sample conditioning other than the addition of ammonium sulfate. Butyl-HIC chromatography exhibited good resolving power and could be run in Tris pH 8 or HEPES pH 7.5. Product yield, however, was low. Chromatograms obtained by butyl-HIC revealed several peaks, more than should have been present given the relative purity of the DEAE-derived starting material. Either DEAE copurified substantial amounts of DNA and protein fragments along with viral particles, or butyl-HIC chromatography was disrupting viral particles. SDS-PAGE analysis of HIC fractions compared with those of DEAE purified viri did not show an excess of proteins. Explanations for this phenomenon could be that the short time in which column-bound ACN53 is subjected to a change in osmotic pressure from 1.5 to 0 M ammonium sulfate causes a disruption of the virion, or that the virions were denatured upon elution due to selective desorption of pieces of the virion as opposed to desorption of the entire particle. HIC did show promise, but needed further development work to resolve the yield and degradation issues.

Immobilized zinc affinity chromatography had several ad-

vantages over both size-exclusion chromatography and HIC for purification of ACN53: IZAC gave higher product recovery and did not require sample manipulation of the DEAE fraction pool prior to loading. Impurities removed by this method eluted in the flowthrough peak and were well resolved from product, leading to simpler pooling criteria. Another advantage of IZAC was that it could be used to reduce the salt concentration of the DEAE pool in a slow, controlled fashion to minimize product loss through osmotic shock damage. IZAC was found to be reproducible, and when used in conjunction with DEAE provided a two-column purification protocol capable of delivering pure ACN53 as specified by SDS-PAGE gels and Westerns, A_{260}/A_{280} ratios, and total virus particle-to-infectious virus particle ratios. Stepwise and overall recovery in terms of total virus particles and infectious virus particles are summarized in Tables 1 and 2.

The stepwise recoveries from the DEAE and IZAC ranged from 44 to 67%. The loss of virus after each step could be attributed to two main factors. First, there was selective pooling of the column fractions. A greater source of loss was the physical trapping of virus in the column resin itself. Virus particles are large enough to be trapped within the resin pores. When the column was cleaned with NaOH, the particles were dissolved and eluted in a degraded form. In optimizing a chromatographic protocol for the purification of virus, column resins must be chosen and tested for the effectiveness of separation due to their chemical functionalities as well as recovery due to their physical architecture.

Analysis of the biological activities of DEAE-IZAC-ACN53 demonstrated that chromatographically produced ACN53 is equal to or better than CsCl-ACN53 in terms of purity and activity. The chromatographic procedure takes less than a day to perform. The ultracentrifugation protocol requires 3 days. A major advantage to the chromatographic procedure is that it can be scaled up using standard, automation-capable procedures, and process-scale chromatography equipment. The CsCl purification method, in contrast, is limited by the capacity of laboratory centrifuges.

The preferred process for the purification of ACN53 is outlined in Fig. 9. Infected cell lysate is treated with nuclease prior to the chromatographic steps. Clarification is then accomplished by step filtration through 0.8- μm followed by 0.2- μm membranes. If necessary, a larger-pore (*e.g.*, 5 μm) prefiltration step can be added for more viscous suspensions. Adjustment to pH 7.5/300 mM NaCl is then performed in preparation for loading onto a DEAE column. The product peak, as detected by the A_{260}/A_{280} -nm ratio or the characteristic photo-diode array spectrum, is pooled and directly injected onto a zinc-charged, iso-osmotically equilibrated metal affinity column. The ionic strength of the buffer is then gradually lowered to approximate phosphate-buffered saline (~ 150 mM NaCl) prior to elution of product with a glycine gradient. This material is then dialyzed into the final formulation.

The use of chromatography for the purification of recombinant adenoviruses for use in gene therapies provides an effective alternative to cesium chloride density gradient ultracentrifugations. There are several advantages related to this methodology, including quality, consistency, decreased process time, system automation, and the ability to process large

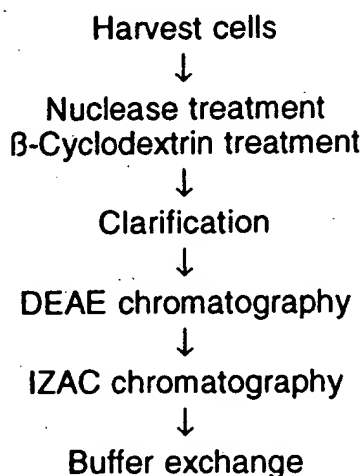


FIG. 9. Process flow diagram for the purification of ACN53. A scalable process for the purification of adenoviral vectors is presented. Details of the process are presented in the Materials and Methods section.

amounts of crude lysate. This method can also be used to purify other recombinant Ad 5. The purification scheme developed specifically for our ACN53 vector selects for product based on the surface characteristics of the virion. These characteristics should not change with different internal DNA constructs, leading to similar chromatographic behaviors independent of the target gene inserted inside the vector. We have begun preliminary investigations into the purification of recombinant Ad 5 vectors containing genes other than human p53 and have found them to behave the same as ACN53.

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REFERENCES

- ALBRECHTSEN, M., and HEIDE, M. (1990). Purification of plant virus coat proteins by high performance liquid chromatography. *J. Virol. Methods* 28, 245-256.
- BARTEK, J., BARTKOVA, J., VOJTESSEK, B., STASKOVA, Z., LUKAS, J., REJTHAR, A., KOVARIK, J., MIDGLEY, C.A., GANNON, J.V., and LANE, D.P. (1991). Aberrant expression of the p53 oncoprotein is a common feature of a wide spectrum of human malignancies. *Oncogene* 6, 1699-1703.
- BELEW, M., YIP, T., ANDERSON, L., and EHRNSTROM, R. (1987). High performance analytical applications of immobilized metal ion affinity chromatography. *Anal. Biochem.* 164, 457-465.
- BRAKKE, M. (1956). Stability of potato yellow-dwarf virus. *Virology* 2, 463-476.
- CROOKS, A., LEE, J., DOWSETT, A., and STEPHENSON, J. (1990). Purification and analysis of infectious virions non native non-structural antigens from cells infected with tick-borne encephalitis virus. *J. Chrom.* 502, 59-68.
- DIACO, R., HILL, J., and DURAND, D. (1986). Purification of soybean mosaic virus by affinity chromatography using monoclonal antibodies. *J. Gen. Virol.* 67, 345-351.
- FOWLER, E., RAAB-TRAUB, N., and HESTER, S. (1985). Purification of biologically active Epstein-Barr virus by affinity chromatography and non-ionic Density gradient centrifugation. *J. Virol. Methods* 11, 59-74.
- GREEN, M., and PINA, M. (1964). Biochemical studies on adenovirus multiplication, VI. Properties of highly purified tumorigenic human adenoviruses and their DNA's. *Proc. Natl. Acad. Sci. USA* 51, 1251-1259.
- GREEN, M., PINA, M., KIMES, R., WENSIK, P., MACHATTIE, L., and THOMAS, C. JR. (1967). Adenovirus DNA: I. Molecular weight and conformation. *Proc. Natl. Acad. Sci. USA* 57, 1302-1309.
- HARUNA, J., YAOSI, H., KONO, R., and WATANABE, I. (1961). Separation of adenovirus by chromatography on DEAE-cellulose. *Virology* 13, 264-267.
- HEWISH, D., and SHUKLA, D. (1983). Purification of Barley Yellow Dwarf virus by gel filtration on Sephacryl® S-1000 superfine. *J. Virol. Methods* 7, 223-228.
- HJORTH, R., and MORENO-LOPEZ, J. (1982). Purification of bovine papilloma virus by gel filtration on Sephacryl® S-1000 Superfine. *J. Virol. Methods* 5, 151-158.
- HOLLSTEIN, M., SIDRANSKY, D., VOGELSTEIN, B., and HARRIS, C. (1991). P53 mutations in human cancers. *Science* 253, 49-53.
- KORWITZ, M.S. (1990). Adenoviridae and their replication. In *Virology*, 2nd ed. B.N. Fields et al., eds. (Raven Press, New York).
- KATO, Y., NAKAMURA, K., and HASIMOTO, T. (1986). High-performance metal chelate affinity chromatography of proteins. *J. Chrom.* 354, 511-517.
- KLEMPERER, H.G., and PEREIRA, H.G. (1959). Study of adenovirus antigen fractionation by chromatography on DEAE cellulose. *Virology* 9, 536-545.
- LARK, K., and ADAMS, M. (1953). The stability of phages as a function of the ionic environment. *Cold Spring Harbor Symposia Quant. Biol.* 18, 171-183.
- LAVER, W.G., YOUNGHUSBAND, H.B., and WRIGLEY, N.G. (1971). Purification and properties of chick embryo lethal orphan virus (an avian adenovirus). *Virology* 45, 598-614.
- LYNN, D. (1992). A BASIC computer program for analyzing endpoint assays. *BioTechniques* 12, 880-881.
- MAIZEL, J. JR., WHITE, D., and SCHARFF, M. (1968). The polypeptides of Adenovirus 1: Evidence for multiple protein components in the virion and a comparison of types 2,7A and 12. *Virology* 36, 115-125.
- MONCANY, M., RÉVET, B., and GIRARD, M. (1980). Characterization of a new adenovirus type 5 assembly intermediate. *J. Gen. Virol.* 50, 33-47.
- NJAYOU, M., and QUASH, G. (1991). Purification of measles virus by affinity chromatography and by ultracentrifugation: a comparative study. *J. Virol. Methods* 32, 67-77.
- OLIVON, M., WALTER, A., and BLUMENTHAL, R. (1986). Sizing and separation of liposomes, biological vesicles and viruses by high performance liquid chromatography. *Anal. Biochem.* 152, 262-274.
- PHILIPSON, L. (1960). Separation on DEAE cellulose of components associated with adenovirus reproduction. *Virology* 10, 459-465.
- PHILIPSON, L. (1961). Adenovirus assay by the fluorescent cell-counting procedure. *Virology* 15, 263-268.

- PHILIPSON, L. (1983). Structure and Assembly of Adenoviruses. *Curr. Topics Microbiol. Immunol.* **109**, 2-52.
- WILLS, K., MANEVAL, D., MENZEL, P., HARRIS, M., SUTJIPTO, S.M. VAILLANCOURT, M., HUANG, W., JOHNSON, D., ANDERSON, S., WEN, S., BOOKSTEIN, R., SHEPARD, M., and GREGORY, R. (1994). Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer. *Hum. Gene Ther.* **5**, 1079-1088.
- VAN DER EB, A., KESTERN, L., and VAN BRUGGEN, E. (1969). Structural properties of adenovirus DNA's. *Biochem. Biophys. Acta* **182**, 530-541.

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(54) Title: AN IMPROVED METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS

(57) Abstract

The present invention addresses the need to improve the yields of viral vectors when grown in cell culture systems. In particular, it has been demonstrated that for adenovirus, the use of low-medium perfusion rates in an attached cell culture system provides for improved yields. In other embodiments, the inventors have shown that there is improved Ad-p53 production with cells grown in serum-free conditions, and in particular in serum-free suspension culture. Also important to the increase of yields is the use of detergent lysis. Combination of these aspects of the invention permits purification of virus by a single chromatography step that results in purified virus of the same quality as preparations from double CsCl banding using an ultracentrifuge.

	Titer (PFU/ml)	Vol. (ml)	Yield (PFU)	Recovery (%)	
				Step	Acc.
Cube (low perfusion rate, keep glucose > 1g/L)					
↓ 1% Tween-20 in buffer A					
Harvest					
↓ Clarification and Filtration (0.22 µm)					
Virus solution	2.6x10 ⁹	1900	4.9x10 ¹²		
↓ Conc./diaf. (10-fold conc., diaf. into 1M NaCl buffer A)					
Conc. sup	2.5x10 ¹⁰	200	5x10 ¹²	102%	
↓ Benzonase treatment (O/N, RT, 100u/ml)					
Treated sup					
↓ Dilute with water to conductivity = 22-25 mS/cm					
Diluted virus solution	7x10 ⁹	700	4.9x10 ¹²	98%	100%
↓					
Purified virus	1.5x10 ¹⁰	240	3.6x10 ¹²	73%	73%
↓ conc./diaf (5-fold conc.)					
Final purified product	7x10 ¹⁰	50	3.5x10 ¹²	97%	71%

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DESCRIPTION

AN IMPROVED METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS

5

BACKGROUND OF THE INVENTION

The present application is a continuation-in-part of co-pending U.S. Provisional Patent Application Serial No. 60/031,329 filed November 20, 1997. The entire text of the above-referenced disclosure is specifically incorporated by reference
10 herein without disclaimer.

1. Field of the Invention

The present invention relates generally to the fields of cell culture and virus production. More particularly, it concerns improved methods for the culturing of
15 mammalian cells, infection of those cells with adenovirus and the production of infectious adenovirus particles therefrom.

2. Description of Related Art

Adenoviral vectors, which express therapeutic proteins, are currently being
20 evaluated in the clinic for the treatment of a variety of cancer indications, including lung and head and neck cancers. As the clinical trials progress, the demand for clinical grade adenoviral vectors is increasing dramatically. The projected annual demand for a 300 patient clinical trial could reach approximately 6×10^{14} PFU.

25 Traditionally, adenoviruses are produced in commercially available tissue culture flasks or "cellfactories." Virus infected cells are harvested and freeze-thawed to release the viruses from the cells in the form of crude cell lysate. The produced crude cell lysate (CCL) is then purified by double CsCl gradient ultracentrifugation. The typically reported virus yield from 100 single tray cellfactories is about 6×10^{12}
30 PFU. Clearly, it becomes unfeasible to produce the required amount of virus using

this traditional process. New scaleable and validatable production and purification processes have to be developed to meet the increasing demand.

The purification throughput of CsCl gradient ultracentrifugation is so limited
5 that it cannot meet the demand for adenoviral vectors for gene therapy applications. Therefore, in order to achieve large scale adenoviral vector production, purification methods other than CsCl gradient ultracentrifugation have to be developed. Reports on the chromatographic purification of viruses are very limited, despite the wide application of chromatography for the purification of recombinant proteins. Size
10 exclusion, ion exchange and affinity chromatography have been evaluated for the purification of retroviruses, tick-borne encephalitis virus, and plant viruses with varying degrees of success (Crooks, *et al.*, 1990; Aboud, *et al.*, 1982; McGrath *et al.*, 1978, Smith and Lee, 1978; O'Neil and Balkovic, 1993). Even less research has been done on the chromatographic purification of adenovirus. This lack of research activity
15 may be partially attributable to the existence of the effective, albeit non-scalable, CsCl gradient ultracentrifugation purification method for adenoviruses.

Recently, Huyghe *et al.* (1996) reported adenoviral vector purification using ion exchange chromatography in conjunction with metal chelate affinity
20 chromatography. Virus purity similar to that from CsCl gradient ultracentrifugation was reported. Unfortunately, only 23% of virus was recovered after the double column purification process. Process factors that contribute to this low virus recovery are the freeze/thaw step utilized by the authors to lyse cells in order to release the virus from the cells and the two column purification procedure.

25

Clearly, there is a demand for an effective and scaleable method of adenoviral vector production that will recover a high yield of product to meet the ever increasing demand for such products.

SUMMARY OF THE INVENTION

The present invention describes a new process for the production and purification of adenovirus. This new production process offers not only scalability
5 and validatability but also virus purity comparable to that achieved using CsCl gradient ultracentrifugation.

Thus the present invention provides a method for producing an adenovirus comprising growing host cells in media at a low perfusion rate, infecting the host cells
10 with an adenovirus, harvesting and lysing the host cells to produce a crude cell lysate, concentrating the crude cell lysate, exchanging buffer of crude cell lysate, and reducing the concentration of contaminating nucleic acids in the crude cell lysate.

In particular embodiments, the method further comprises isolating an
15 adenoviral particle from the lysate using chromatography. In certain embodiments, the isolating consists essentially of a single chromatography step. In other embodiments, the chromatography step is ion exchange chromatography. In particularly preferred embodiments, the ion exchange chromatography is carried out at a pH range of between about 7.0 and about 10.0. In more preferred embodiments, the
20 ion exchange chromatography is anion exchange chromatography. In certain embodiments the anion exchange chromatography utilizes DEAE, TMAE, QAE, or PEI. In other preferred embodiments, the anion exchange chromatography utilizes Toyopearl Super Q 650M, MonoQ, Source Q or Fractogel TMAE.

25 In certain embodiments of the present invention the glucose concentration in the media is maintained between about 0.7 and about 1.7g/L. In certain other embodiments, the exchanging buffer involves a diafiltration step.

In preferred embodiments of the present invention, the adenovirus comprises
30 an adenoviral vector encoding an exogenous gene construct. In certain such embodiments, the gene construct is operatively linked to a promoter. In particular

embodiments, the promoter is SV40 IE, RSV LTR, β -actin or CMV IE, adenovirus major late, polyoma F9-1, or tyrosinase. In particular embodiments of the present invention, the adenovirus is a replication-incompetent adenovirus. In other embodiments, the adenovirus is lacking at least a portion of the E1-region. In certain
5 aspects, the adenovirus is lacking at least a portion of the E1A and/or E1B region. In other embodiments, the host cells are capable of complementing replication. In particularly preferred embodiments, the host cells are 293 cells.

In preferred a embodiment of the present invention it is contemplated that the
10 exogenous gene construct encodes a therapeutic gene. For example, the therapeutic gene may encode antisense *ras*, antisense *myc*, antisense *raf*, antisense *erb*, antisense *src*, antisense *fms*, antisense *jun*, antisense *trk*, antisense *ret*, antisense *gsp*, antisense *hst*, antisense *bcl* antisense *abl*, Rb, CFTR, p16, p21, p27, p57, p73, C-CAM, APC, CTS-1, *zac1*, scFV *ras*, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL,
15 MMAC1, FCC, MCC, BRCA2, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, GM-CSF G-CSF, thymidine kinase or p53.

In certain aspects of the present invention, the cells may be harvested and lysed *ex situ* using a hypotonic solution, hypertonic solution, freeze-thaw, sonication,
20 impinging jet, microfluidization or a detergent. In other aspects, the cells are harvested and lysed *in situ* using a hypotonic solution, hypertonic solution, or a detergent. As used herein the term "in situ" refers to the cells being located within the tissue culture apparatus for example CellCube™ and "ex situ" refers to the cells being removed from the tissue culture apparatus.

25

In particular embodiments, the cells are lysed and harvested using detergent. In preferred embodiments the detergent may be Thesit®, NP-40®, Tween-20®, Brij-58®, Triton X®-100 or octyl glucoside. In other aspects of the present invention lysis is achieved through autolysis of infected cells. In certain other aspects of the present
30 invention the cell lysate is treated with Benzonase®, or Pulmozyme®.

In particular embodiments, the method further comprises a concentration step employing membrane filtration. In particular embodiments, the filtration is tangential flow filtration. In preferred embodiments, the filtration may utilize a 100 to 300K NMWC, regenerated cellulose, or polyether sulfone membrane.

5

The present invention also provides an adenovirus produced according to a process comprising the steps of growing host cells in media at a low perfusion rate, infecting the host cells with an adenovirus, harvesting and lysing the host cells to produce a crude cell lysate, concentrating the crude cell lysate, exchanging buffer of
10 crude cell lysate, and reducing the concentration of contaminating nucleic acids in the crude cell lysate.

Other aspects of the present invention provide a method for the purification of an adenovirus comprising growing host cells, infecting the host cells with an
15 adenovirus, harvesting and lysing the host cells by contacting the cells with a detergent to produce a crude cell lysate, concentrating the crude cell lysate, exchanging buffer of crude cell lysate, and reducing the concentration of contaminating nucleic acids in the crude cell lysate.

20 In particular embodiments, the detergent may be Thesit[®], NP-40[®], Tween-20[®], Brij-58[®], Triton X-100[®] or octyl glucoside. In more particular embodiments the detergent is present in the lysis solution at a concentration of about 1% (w/v).

In other aspects of the present invention there is provided an adenovirus
25 produced according to a process comprising the steps of growing host cells, infecting the host cells with an adenovirus, harvesting and lysing the host cells by contacting the cells with a detergent to produce a crude cell lysate, concentrating the crude cell lysate, exchanging buffer of crude cell lysate, and reducing the concentration of contaminating nucleic acids in the crude cell lysate.

30

In yet another embodiment, the present invention provides a method for the purification of an adenovirus comprising the steps of growing host cells in serum-free media; infecting said host cells with an adenovirus; harvesting and lysing said host cells to produce a crude cell lysate; concentrating said crude cell lysate; exchanging
5 buffer of crude cell lysate; and reducing the concentration of contaminating nucleic acids in said crude cell lysate. In preferred embodiments, the cells may be grown independently as a cell suspension culture or as an anchorage-dependent culture.

In particular embodiments, the host cells are adapted for growth in serum-free
10 media. In more preferred embodiments, the adaptation for growth in serum-free media comprises a sequential decrease in the fetal bovine serum content of the growth media. More particularly, the serum-free media comprises a fetal bovine serum content of less than 0.03% v/v.

15 In other embodiments, the method further comprises isolating an adenoviral particle from said lysate using chromatography. In preferred embodiments, the isolating consists essentially of a single chromatography step. More particularly, the chromatography step is ion exchange chromatography.

20 Also contemplated by the present invention is an adenovirus produced according to a process comprising the steps of growing host cells in serum-free media; infecting said host cells with an adenovirus; harvesting and lysing said host cells to produce a crude cell lysate; concentrating said crude cell lysate; exchanging buffer of crude cell lysate; and, reducing the concentration of contaminating nucleic acids in
25 said crude cell lysate.

The present invention further provides a 293 host cell adapted for growth in serum-free media. In certain aspects, the adaptation for growth in serum-free media comprises a sequential decrease in the fetal bovine serum content of the growth
30 media. In particular embodiments, the cell is adapted for growth in suspension culture. In particular embodiments, the cells of the present invention are designated

IT293SF cells. These cells were deposited with the American Tissue Culture Collection (ATCC) in order to meet the requirements of the Budapest Treaty on the international recognition of deposits of microorganisms for the purposes of patent procedure. The cells were deposited by Dr. Shuyuan Zhang on behalf of Introgen Therapeutics, Inc. (Houston, Tx.), on November 17, 1997. IT293SF cell line is derived from an adaptation of 293 cell line into serum free suspension culture as described herein. The cells may be cultured in IS 293 serum-free media (Irvine Scientific, Santa Ana, Ca.) supplemented with 100mg/L heparin and 0.1% pluronic F-68, and are permissive to human adenovirus infection.

10

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

15

BRIEF DESCRIPTION OF THE DRAWINGS

20

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

25

FIG. 1A and FIG. 1B. HPLC profiles of the viral solutions from production runs using medium perfusion rates characterized as "high" (FIG. 1A) and "low" (FIG. 1B).

30

FIG. 2. The HPLC profile of crude cell lysate (CCL) from CellCube™ (solid line A₂₆₀; dotted line A₂₈₀).

FIG. 3A, FIG. 3B, FIG 3C, FIG. 3D and FIG. 3E. The HPLC profiles of lysis solutions from CellCube™ using different detergents. FIG. 3A Thesit®. FIG. 3B Triton®X-100. FIG. 3C. NP-40®. FIG. 3D. Brij®80. FIG. 3E. Tween®20.
5 Detergent concentration: 1% (w/v) lysis temperature: room temperature. (solid line A₂₆₀; dotted line A₂₈₀).

FIG. 4A and FIG. 4B. The HPLC profiles of virus solution before (FIG. 4A) and after (FIG. 4B) Benzonase treatment. (solid line A₂₆₀; dotted line A₂₈₀).

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FIG. 5. The HPLC profile of virus solution after Benzonase treatment in the presence of 1M NaCl. (solid line A₂₆₀; dotted line A₂₈₀).

FIG. 6. Purification of AdCMVp53 virus under buffer A condition of 20mM
15 Tris + 1mM MgCl₂ + 0.2M NaCl, pH=7.5.

FIG. 7. Purification of AdCMVp53 virus under buffer A condition of 20mM Tris + 1mM MgCl₂ + 0.2M NaCl, pH=9.0.

20 **FIG. 8A, FIG. 8B, and FIG. 8C.** HPLC analysis of fractions obtained during purification FIG. 8A fraction 3. FIG. 8B fraction 4, FIG. 8C fraction 8. (solid line A₂₆₀; dotted line A₂₈₀).

FIG. 9. Purification of AdCMVp53 virus under buffer A condition of 20mM
25 Tris + 1mM MgCl₂ + 0.3M NaCl, pH=9.

FIG. 10A, FIG. 10B, FIG. 10C, FIG. 10D and FIG. 10E. HPLC analysis of crude virus fractions obtained during purification and CsCl gradient purified virus. FIG. 10A Crude virus solution. FIG. 10B Flow through. FIG. 10C. Peak number 1.
30 FIG. 10D. Peak number 2. FIG. 10E. CsCl purified virus. (solid line A₂₆₀; dotted line A₂₈₀).

FIG. 11. HPLC purification profile from a 5cm id column.

FIG. 12. The major adenovirus structure proteins detected on SDS-PAGE.

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FIG. 13. The BSA concentration in the purified virus as detected level of the western blot assay.

FIG. 14. The chromatogram for the crude cell lysate material generated from the CellCube™.

10

FIG. 15. The elution profile of treated virus solution purified using the method of the present invention using Toyopearl SuperQ resin.

FIG. 16A and FIG. 16B. HPLC analysis of virus fraction from purification protocol. FIG 16A HPLC profiles of virus fraction from first purification step. FIG.16B HPLC profiles of virus fraction from second purification. (solid line A_{260} ; dotted line A_{280}).

15

FIG. 17. Purification of 1% Tween® harvest virus solution under low medium perfusion rate.

20

FIG. 18. HPLC analysis of the virus fraction produced under low medium perfusion rate.

25

FIG. 19A, FIG. 19B and FIG. 19C. Analysis of column purified virus. FIG. 19A SDS-PAGE analysis. FIG. 19B Western blot for BSA. FIG. 19C nucleic acid slot blot to determine the contaminating nucleic acid concentration.

FIG. 20A, FIG. 20B, FIG. 20C, FIG. 20D, FIG. 20E and FIG. 20F. Capacity study of the Toyopearl SuperQ 650M resin. FIG. 20A Flow through from

30

loading ratio of 1:1. FIG. 20B. Purified virus from loading ratio of 1:1. FIG. 20C Flow through of loading ratio of 2:1. FIG. 20D. Purified virus from the loading ratio of 2:1. FIG. 20E Flow through from loading ratio of 3:1. FIG. 20F. Purified virus from the loading ratio of 3:1. (solid line A_{260} ; dotted line A_{280}).

5

FIG. 21. Isopycnic CsCl ultracentrifugation column purified virus.

FIG. 22. The HPLC profiles of intact viruses present in the column purified virus. A. Intact virus B. Defective virus. (solid line A_{260} ; dotted line A_{280}).

10

FIG. 23. A production and purification flow chart for AdCMVp53

DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

15 It has been shown that adenoviral vectors can successfully be used in eukaryotic gene expression and vaccine development. Recently, animal studies have demonstrated that recombinant adenovirus could be used for gene therapy. Successful studies in administering recombinant adenovirus to different tissues have proven the effectiveness of adenoviral vectors in therapy. This success has led to the use of such
20 vectors in human clinical trials. There now is an increased demand for the production of adenoviral vectors to be used in various therapies. The techniques currently available are insufficient to meet such a demand. The present invention provides methods for the production of large amounts of adenovirus for use in such therapies.

25 The present invention involves a process that has been developed for the production and purification of a replication deficient recombinant adenovirus. The production process is based on the use of a Cellcube™ bioreactor for cell growth and virus production. It was found that a given perfusion rate, used during cell growth and the virus production phases of culturing, has a significant effect on the
30 downstream purification of the virus. More specifically, a low to medium perfusion rate improves virus production. In addition, lysis solution composed of buffered

detergent, used to lyse cells in the Cellcube™ at the end of virus production phase, also improves the process. With these two advantages, the harvested crude virus solution can be purified using a single ion exchange chromatography run, after concentration/diafiltration and nuclease treatment to reduce the contaminating nucleic acid concentration in the crude virus solution. The column purified virus has equivalent purity relative to that of double CsCl gradient purified virus. The total process recovery of the virus product is 70% ± 10%. This is a significant improvement over the results reported by Huyghe *et al.* (1996). Compared to double CsCl gradient ultracentrifugation, column purification has the advantage of being more consistent, scaleable, validatable, faster and less expensive. This new process represents a significant improvement in the technology for manufacturing of adenoviral vectors for gene therapy.

Therefore, the present invention is designed to take advantage of these improvements in large scale culturing systems and purification for the purpose of producing and purifying adenoviral vectors. The various components for such a system, and methods of producing adenovirus therewith, are set forth in detail below.

1. Host Cells

A) Cells

In a preferred embodiment, the generation and propagation of the adenoviral vectors depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by Adenovirus serotype 5 (Ad5) DNA fragments and constitutively expresses E1 proteins (Graham *et al.*, 1977). Since the E3 region is dispensable from the Ad genome (Jones and Shenk, 1978), the current Ad vectors, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, 1991; Bett *et al.*, 1994).

A first aspect of the present invention is the recombinant cell lines which express part of the adenoviral genome. These cells lines are capable of supporting replication of adenovirus recombinant vectors and helper viruses having defects in

certain adenoviral genes, *i.e.*, are "permissive" for growth of these viruses and vectors. The recombinant cell also is referred to as a helper cell because of the ability to complement defects in, and support replication of, replication-incompetent adenoviral vectors. The prototype for an adenoviral helper cell is the 293 cell line, which
5 contains the adenoviral E1 region. 293 cells support the replication of adenoviral vectors lacking E1 functions by providing *in trans* the E1-active elements necessary for replication.

Helper cells according to the present invention are derived from a mammalian
10 cell and, preferably, from a primate cell such as human embryonic kidney cell. Although various primate cells are preferred and human or even human embryonic kidney cells are most preferred, any type of cell that is capable of supporting replication of the virus would be acceptable in the practice of the invention. Other cell types might include, but are not limited to Vero cells, CHO cells or any
15 eukaryotic cells for which tissue culture techniques are established as long as the cells are adenovirus permissive. The term "adenovirus permissive" means that the adenovirus or adenoviral vector is able to complete the entire intracellular virus life cycle within the cellular environment.

20 The helper cell may be derived from an existing cell line, *e.g.*, from a 293 cell line, or developed *de novo*. Such helper cells express the adenoviral genes necessary to complement *in trans* deletions in an adenoviral genome or which supports replication of an otherwise defective adenoviral vector, such as the E1, E2, E4, E5 and late functions. A particular portion of the adenovirus genome, the E1 region, has
25 already been used to generate complementing cell lines. Whether integrated or episomal, portions of the adenovirus genome lacking a viral origin of replication, when introduced into a cell line, will not replicate even when the cell is superinfected with wild-type adenovirus. In addition, because the transcription of the major late unit is after viral DNA replication, the late functions of adenovirus cannot be
30 expressed sufficiently from a cell line. Thus, the E2 regions, which overlap with late

functions (L1-5), will be provided by helper viruses and not by the cell line. Typically, a cell line according to the present invention will express E1 and/or E4.

As used herein, the term "recombinant" cell is intended to refer to a cell into which a gene, such as a gene from the adenoviral genome or from another cell, has been introduced. Therefore, recombinant cells are distinguishable from naturally-occurring cells which do not contain a recombinantly-introduced gene. Recombinant cells are thus cells having a gene or genes introduced through "the hand of man."

Replication is determined by contacting a layer of uninfected cells, or cells infected with one or more helper viruses, with virus particles, followed by incubation of the cells. The formation of viral plaques, or cell free areas in the cell layer, is the result of cell lysis caused by the expression of certain viral products. Cell lysis is indicative of viral replication.

Examples of other useful mammalian cell lines that may be used with a replication competent virus or converted into complementing host cells for use with replication deficient virus are Vero and HeLa cells and cell lines of Chinese hamster ovary, W138, BHK, COS-7, HepG2, 3T3, RIN and MDCK cells.

B) Growth in selection media

In certain embodiments, it may be useful to employ selection systems that preclude growth of undesirable cells. This may be accomplished by virtue of permanently transforming a cell line with a selectable marker or by transducing or infecting a cell line with a viral vector that encodes a selectable marker. In either situation, culture of the transformed/transduced cell with an appropriate drug or selective compound will result in the enhancement, in the cell population, of those cells carrying the marker.

Examples of markers include, but are not limited to, HSV thymidine kinase, hypoxanthine-guanine phosphoribosyltransferase and adenine

phosphoribosyltransferase genes, in *tk-*, *hgpri-* or *apri-* cells, respectively. Also, anti-metabolite resistance can be used as the basis of selection for *dhfr*, that confers resistance to methotrexate; *gpt*, that confers resistance to mycophenolic acid; *neo*, that confers resistance to the aminoglycoside G418; and *hygro*, that confers resistance to hygromycin.

C) Growth in serum weaning

Serum weaning adaptation of anchorage-dependent cells into serum-free suspension cultures have been used for the production of recombinant proteins (Berg, 1993) and viral vaccines (Perrin, 1995). There have been few reports on the adaptation of 293A cells into serum-free suspension cultures until recently. Gilbert reported the adaptation of 293A cells into serum-free suspension cultures for adenovirus and recombinant protein production (Gilbert, 1996). Similar adaptation method had been used for the adaptation of A549 cells into serum-free suspension culture for adenovirus production (Morris *et al.*, 1996). Cell-specific virus yields in the adapted suspension cells, however, are about 5-10-fold lower than those achieved in the parental attached cells.

Using the similar serum weaning procedure, the inventors have successfully adapted the 293A cells into serum-free suspension culture (293SF cells). In this procedure, the 293 cells were adapted to a commercially available 293 media by sequentially lowering down the FBS concentration in T-flasks. Briefly, the initial serum concentration in the media was approximately 10% FBS DMEM media in T-75 flask and the cells were adapted to serum-free IS 293 media in T-flasks by lowering down the FBS concentration in the media sequentially. After 6 passages in T-75 flasks the FBS% was estimated to be about 0.019% and the 293 cells. The cells were subcultured two more times in the T flasks before they were transferred to spinner flasks. The results described herein below show that cells grow satisfactorily in the serum-free medium (IS293 medium, Irvine Scientific, Santa Ana, CA). Average doubling time of the cells were 18-24 h achieving stationary cell concentrations in the order of $4-10 \times 10^6$ cells/ml without medium exchange.

D) Adaptation of cells for Suspension Culture

Two methodologies have been used to adapt 293 cells into suspension cultures. Graham adapted 293A cells into suspension culture (293N3S cells) by 3 serial passages in nude mice (Graham, 1987). The suspension 293N3S cells were
5 found to be capable of supporting E1⁻ adenoviral vectors. However, Garnier *et al.* (1994) observed that the 293N35 cells had a relatively long initial lag phase in suspension, a low growth rate, and a strong tendency to clump.

10 The second method that has been used is a gradual adaptation of 293A cells into suspension growth (Cold Spring Harbor Laboratories, 293S cells). Garnier *et al.* (1994) reported the use of 293S cells for production of recombinant proteins from adenoviral vectors. The authors found that 293S cells were much less clumpy in calcium-free media and a fresh medium exchange at the time of virus infection could
15 significantly increase the protein production. It was found that glucose was the limiting factor in culture without medium exchange.

In the present invention, the 293 cells adapted for growth in serum-free conditions were adapted into a suspension culture. The cells were transferred in a
20 serum-free 250 mL spinner suspension culture (100 mL working volume) for the suspension culture at an initial cell density of between about 1.18×10^5 vc/mL and about 5.22×10^5 vc/mL. The media may be supplemented with heparin to prevent aggregation of cells. This cell culture systems allows for some increase of cell density whilst cell viability is maintained. Once these cells are growing in culture, they cells
25 are subcultured in the spinner flasks approximately 7 more passages. It may be noted that the doubling time of the cells is progressively reduced until at the end of the successive passages the doubling time is about 1.3 day, *i.e.* comparable to 1.2 day of the cells in 10% FBS media in the attached cell culture. In the serum-free IS 293 media supplemented with heparin almost all the cells existed as individual cells not
30 forming aggregates of cells in the suspension culture.

2. Cell Culture Systems

The ability to produce infectious viral vectors is increasingly important to the pharmaceutical industry, especially in the context of gene therapy. Over the last decade, advances in biotechnology have led to the production of a number of important viral vectors that have potential uses as therapies, vaccines and protein production machines. The use of viral vectors in mammalian cultures has advantages over proteins produced in bacterial or other lower lifeform hosts in their ability to post-translationally process complex protein structures such as disulfide-dependent folding and glycosylation.

10

Development of cell culture for production of virus vectors has been greatly aided by the development in molecular biology of techniques for design and construction of vector systems highly efficient in mammalian cell cultures, a battery of useful selection markers, gene amplification schemes and a more comprehensive understanding of the biochemical and cellular mechanisms involved in procuring the final biologically-active molecule from the introduced vector.

Frequently, factors which affect the downstream (in this case, beyond the cell lysis) side of manufacturing scale-up were not considered before selecting the cell line as the host for the expression system. Also, development of bioreactor systems capable of sustaining very high density cultures for prolonged periods of time have not lived up to the increasing demand for increased production at lower costs.

The present invention will take advantage of the recently available bioreactor technology. Growing cells according to the present invention in a bioreactor allows for large scale production of fully biologically-active cells capable of being infected by the adenoviral vectors of the present invention. By operating the system at a low perfusion rate and applying a different scheme for purification of the infecting particles, the invention provides a purification strategy that is easily scaleable to produce large quantities of highly purified product.

30

Bioreactors have been widely used for the production of biological products from both suspension and anchorage dependent animal cell cultures. The most widely used producer cells for adenoviral vector production are anchorage dependent human embryonic kidney cells (293 cells). Bioreactors to be developed for adenoviral vector production should have the characteristic of high volume-specific culture surface area in order to achieve high producer cell density and high virus yield. Microcarrier cell culture in stirred tank bioreactor provides very high volume-specific culture surface area and has been used for the production of viral vaccines (Griffiths, 1986). Furthermore, stirred tank bioreactors have industrially been proven to be scaleable. The multiplate Cellcube™ cell culture system manufactured by Corning-Costar also offers a very high volume-specific culture surface area. Cells grow on both sides of the culture plates hermetically sealed together in the shape of a compact cube. Unlike stirred tank bioreactors, the Cellcube™ culture unit is disposable. This is very desirable at the early stage production of clinical product because of the reduced capital expenditure, quality control and quality assurance costs associated with disposable systems. In consideration of the advantages offered by the different systems, both the stirred tank bioreactor and the Cellcube™ system were evaluated for the production of adenovirus.

20 A) *Anchorage-dependent versus non-anchorage-dependent cultures.*

Animal and human cells can be propagated *in vitro* in two modes: as non-anchorage dependent cells growing freely in suspension throughout the bulk of the culture; or as anchorage-dependent cells requiring attachment to a solid substrate for their propagation (*i.e.*, a monolayer type of cell growth).

25

Non-anchorage dependent or suspension cultures from continuous established cell lines are the most widely used means of large scale production of cells and cell products. Large scale suspension culture based on microbial (bacterial and yeast) fermentation technology has clear advantages for the manufacturing of mammalian cell products. The processes are relatively simple to operate and straightforward to scale up. Homogeneous conditions can be provided in the reactor which allows for

30

precise monitoring and control of temperature, dissolved oxygen, and pH, and ensure that representative samples of the culture can be taken.

However, suspension cultured cells cannot always be used in the production of
5 biologicals. Suspension cultures are still considered to have tumorigenic potential and
thus their use as substrates for production put limits on the use of the resulting
products in human and veterinary applications (Petricciani, 1985; Larsson, 1987).
Viruses propagated in suspension cultures as opposed to anchorage-dependent
cultures can sometimes cause rapid changes in viral markers, leading to reduced
10 immunogenicity (Bahnemann, 1980). Finally, sometimes even recombinant cell lines
can secrete considerably higher amounts of products when propagated as anchorage-
dependent cultures as compared with the same cell line in suspension (Nilsson and
Mosbach, 1987). For these reasons, different types of anchorage-dependent cells are
used extensively in the production of different biological products.

15

B) Reactors and processes for suspension.

Large scale suspension culture of mammalian cultures in stirred tanks was
undertaken. The instrumentation and controls for bioreactors adapted, along with the
design of the fermentors, from related microbial applications. However,
20 acknowledging the increased demand for contamination control in the slower growing
mammalian cultures, improved aseptic designs were quickly implemented, improving
dependability of these reactors. Instrumentation and controls are basically the same as
found in other fermentors and include agitation, temperature, dissolved oxygen, and
pH controls. More advanced probes and autoanalyzers for on-line and off-line
25 measurements of turbidity (a function of particles present), capacitance (a function of
viable cells present), glucose/lactate, carbonate/bicarbonate and carbon dioxide are
available. Maximum cell densities obtainable in suspension cultures are relatively
low at about $2-4 \times 10^6$ cells/ml of medium (which is less than 1 mg dry cell weight
per ml), well below the numbers achieved in microbial fermentation.

30

Two suspension culture reactor designs are most widely used in the industry due to their simplicity and robustness of operation - the stirred reactor and the airlift reactor. The stirred reactor design has successfully been used on a scale of 8000 liter capacity for the production of interferon (Phillips *et al.*, 1985; Mizrahi, 1983). Cells
5 are grown in a stainless steel tank with a height-to-diameter ratio of 1:1 to 3:1. The culture is usually mixed with one or more agitators, based on bladed disks or marine propeller patterns. Agitator systems offering less shear forces than blades have been described. Agitation may be driven either directly or indirectly by magnetically coupled drives. Indirect drives reduce the risk of microbial contamination through
10 seals on stirrer shafts.

The airlift reactor, also initially described for microbial fermentation and later adapted for mammalian culture, relies on a gas stream to both mix and oxygenate the culture. The gas stream enters a riser section of the reactor and drives circulation.
15 Gas disengages at the culture surface, causing denser liquid free of gas bubbles to travel downward in the downcomer section of the reactor. The main advantage of this design is the simplicity and lack of need for mechanical mixing. Typically, the height-to-diameter ratio is 10:1. The airlift reactor scales up relatively easily, has good mass transfer of gasses and generates relatively low shear forces.

20

Most large-scale suspension cultures are operated as batch or fed-batch processes because they are the most straightforward to operate and scale up. However, continuous processes based on chemostat or perfusion principles are available.

25

A batch process is a closed system in which a typical growth profile is seen. A lag phase is followed by exponential, stationary and decline phases. In such a system, the environment is continuously changing as nutrients are depleted and metabolites accumulate. This makes analysis of factors influencing cell growth and productivity,
30 and hence optimization of the process, a complex task. Productivity of a batch process may be increased by controlled feeding of key nutrients to prolong the growth

cycle. Such a fed-batch process is still a closed system because cells, products and waste products are not removed.

In what is still a closed system, perfusion of fresh medium through the culture can be achieved by retaining the cells with a variety of devices (*e.g.* fine mesh spin filter, hollow fiber or flat plate membrane filters, settling tubes). Spin filter cultures can produce cell densities of approximately 5×10^7 cells/ml. A true open system and the simplest perfusion process is the chemostat in which there is an inflow of medium and an outflow of cells and products. Culture medium is fed to the reactor at a predetermined and constant rate which maintains the dilution rate of the culture at a value less than the maximum specific growth rate of the cells (to prevent washout of the cell mass from the reactor). Culture fluid containing cells and cell products and byproducts is removed at the same rate.

15 **C) *Non-perfused attachment systems.***

Traditionally, anchorage-dependent cell cultures are propagated on the bottom of small glass or plastic vessels. The restricted surface-to-volume ratio offered by classical and traditional techniques, suitable for the laboratory scale, has created a bottleneck in the production of cells and cell products on a large scale. In an attempt to provide systems that offer large accessible surfaces for cell growth in small culture volume, a number of techniques have been proposed: the roller bottle system, the stack plates propagator, the spiral film bottles, the hollow fiber system, the packed bed, the plate exchanger system, and the membrane tubing reel. Since these systems are non-homogeneous in their nature, and are sometimes based on multiple processes, they suffer from the following shortcomings - limited potential for scale-up, difficulties in taking cell samples, limited potential for measuring and controlling key process parameters and difficulty in maintaining homogeneous environmental conditions throughout the culture.

30 Despite these drawbacks, a commonly used process for large scale anchorage-dependent cell production is the roller bottle. Being little more than a large,

differently shaped T-flask, simplicity of the system makes it very dependable and, hence, attractive. Fully automated robots are available that can handle thousands of roller bottles per day, thus eliminating the risk of contamination and inconsistency associated with the otherwise required intense human handling. With frequent media
5 changes, roller bottle cultures can achieve cell densities of close to 0.5×10^6 cells/cm² (corresponding to approximately 10^9 cells/bottle or almost 10^7 cells/ml of culture media).

D) *Cultures on microcarriers*

10 In an effort to overcome the shortcomings of the traditional anchorage-dependent culture processes, van Wezel (1967) developed the concept of the microcarrier culturing systems. In this system, cells are propagated on the surface of small solid particles suspended in the growth medium by slow agitation. Cells attach to the microcarriers and grow gradually to confluency on the microcarrier surface. In
15 fact, this large scale culture system upgrades the attachment dependent culture from a single disc process to a unit process in which both monolayer and suspension culture have been brought together. Thus, combining the necessary surface for a cell to grow with the advantages of the homogeneous suspension culture increases production.

20 The advantages of microcarrier cultures over most other anchorage-dependent, large-scale cultivation methods are several fold. First, microcarrier cultures offer a high surface-to-volume ratio (variable by changing the carrier concentration) which leads to high cell density yields and a potential for obtaining highly concentrated cell products. Cell yields are up to $1-2 \times 10^7$ cells/ml when cultures are propagated in a
25 perfused reactor mode. Second, cells can be propagated in one unit process vessels instead of using many small low-productivity vessels (*i.e.*, flasks or dishes). This results in far better nutrient utilization and a considerable saving of culture medium. Moreover, propagation in a single reactor leads to reduction in need for facility space and in the number of handling steps required per cell, thus reducing labor cost and risk
30 of contamination. Third, the well-mixed and homogeneous microcarrier suspension culture makes it possible to monitor and control environmental conditions (*e.g.*, pH,

pO₂, and concentration of medium components), thus leading to more reproducible cell propagation and product recovery. Fourth, it is possible to take a representative sample for microscopic observation, chemical testing, or enumeration. Fifth, since microcarriers settle out of suspension quickly, use of a fed-batch process or harvesting of cells can be done relatively easily. Sixth, the mode of the anchorage-dependent culture propagation on the microcarriers makes it possible to use this system for other cellular manipulations, such as cell transfer without the use of proteolytic enzymes, cocultivation of cells, transplantation into animals, and perfusion of the culture using decanters, columns, fluidized beds, or hollow fibers for microcarrier retainment. Seventh, microcarrier cultures are relatively easily scaled up using conventional equipment used for cultivation of microbial and animal cells in suspension.

E) Microencapsulation of mammalian cells

One method which has shown to be particularly useful for culturing mammalian cells is microencapsulation. The mammalian cells are retained inside a semipermeable hydrogel membrane. A porous membrane is formed around the cells permitting the exchange of nutrients, gases, and metabolic products with the bulk medium surrounding the capsule. Several methods have been developed that are gentle, rapid and non-toxic and where the resulting membrane is sufficiently porous and strong to sustain the growing cell mass throughout the term of the culture. These methods are all based on soluble alginate gelled by droplet contact with a calcium-containing solution. Lim (1982, US Patent 4,352,883, incorporated herein by reference,) describes cells concentrated in an approximately 1% solution of sodium alginate which are forced through a small orifice, forming droplets, and breaking free into an approximately 1% calcium chloride solution. The droplets are then cast in a layer of polyamino acid that ionically bonds to the surface alginate. Finally the alginate is reliquefied by treating the droplet in a chelating agent to remove the calcium ions. Other methods use cells in a calcium solution to be dropped into a alginate solution, thus creating a hollow alginate sphere. A similar approach involves cells in a chitosan solution dropped into alginate, also creating hollow spheres.

Microencapsulated cells are easily propagated in stirred tank reactors and, with beads sizes in the range of 150-1500 μm in diameter, are easily retained in a perfused reactor using a fine-meshed screen. The ratio of capsule volume to total media volume can be maintained from as dense as 1:2 to 1:10. With intracapsular cell densities of up to 10^8 , the effective cell density in the culture is $1-5 \times 10^7$.

The advantages of microencapsulation over other processes include the protection from the deleterious effects of shear stresses which occur from sparging and agitation, the ability to easily retain beads for the purpose of using perfused systems, scale up is relatively straightforward and the ability to use the beads for implantation.

The current invention includes cells which are anchorage-dependent in nature. 293 cells, for example, are anchorage-dependent, and when grown in suspension, the cells will attach to each other and grow in clumps, eventually suffocating cells in the inner core of each clump as they reach a size that leaves the core cells unsustainable by the culture conditions. Therefore, an efficient means of large-scale culture of anchorage-dependent cells is needed in order to effectively employ these cells to generate large quantities of adenovirus.

F) Perfused attachment systems

Perfused attachment systems are a preferred form of the present invention. Perfusion refers to continuous flow at a steady rate, through or over a population of cells (of a physiological nutrient solution). It implies the retention of the cells within the culture unit as opposed to continuous-flow culture which washes the cells out with the withdrawn media (*e.g.*, chemostat). The idea of perfusion has been known since the beginning of the century, and has been applied to keep small pieces of tissue viable for extended microscopic observation. The technique was initiated to mimic the cells milieu *in vivo* where cells are continuously supplied with blood, lymph, or other body fluids. Without perfusion, cells in culture go through alternating phases of

being fed and starved, thus limiting full expression of their growth and metabolic potential.

The current use of perfused culture is in response to the challenge of growing
5 cells at high densities (*i.e.*, $0.1-5 \times 10^8$ cells/ml). In order to increase densities beyond
 $2-4 \times 10^6$ cells/ml, the medium has to be constantly replaced with a fresh supply in
order to make up for nutritional deficiencies and to remove toxic products. Perfusion
allows for a far better control of the culture environment (pH, pO_2 , nutrient levels,
etc.) and is a means of significantly increasing the utilization of the surface area
10 within a culture for cell attachment.

The development of a perfused packed-bed reactor using a bed matrix of a
non-woven fabric has provided a means for maintaining a perfusion culture at
densities exceeding 10^8 cells/ml of the bed volume (CelliGen™, New Brunswick
15 Scientific, Edison, NJ; Wang *et al.*, 1992; Wang *et al.*, 1993; Wang *et al.*, 1994).
Briefly described, this reactor comprises an improved reactor for culturing of both
anchorage- and non-anchorage-dependent cells. The reactor is designed as a packed
bed with a means to provide internal recirculation. Preferably, a fiber matrix carrier is
placed in a basket within the reactor vessel. A top and bottom portion of the basket
20 has holes, allowing the medium to flow through the basket. A specially designed
impeller provides recirculation of the medium through the space occupied by the fiber
matrix for assuring a uniform supply of nutrient and the removal of wastes. This
simultaneously assures that a negligible amount of the total cell mass is suspended in
the medium. The combination of the basket and the recirculation also provides a
25 bubble-free flow of oxygenated medium through the fiber matrix. The fiber matrix is
a non-woven fabric having a "pore" diameter of from 10 μm to 100 μm , providing for
a high internal volume with pore volumes corresponding to 1 to 20 times the volumes
of individual cells.

30 In comparison to other culturing systems, this approach offers several
significant advantages. With a fiber matrix carrier, the cells are protected against

mechanical stress from agitation and foaming. The free medium flow through the basket provides the cells with optimum regulated levels of oxygen, pH, and nutrients. Products can be continuously removed from the culture and the harvested products are free of cells and can be produced in low-protein medium which facilitates subsequent purification steps. Also, the unique design of this reactor system offers an easier way to scale up the reactor. Currently, sizes up to 30 liter are available. One hundred liter and 300 liter versions are in development and theoretical calculations support up to a 1000 liter reactor. This technology is explained in detail in WO 94/17178 (August 4, 1994, Freedman *et al.*), which is hereby incorporated by reference in its entirety.

10

The Cellcube™ (Corning-Costar) module provides a large styrenic surface area for the immobilization and growth of substrate attached cells. It is an integrally encapsulated sterile single-use device that has a series of parallel culture plate joined to create thin sealed laminar flow spaces between adjacent plates.

15

The Cellcube™ module has inlet and outlet ports that are diagonally opposite each other and help regulate the flow of media. During the first few days of growth the culture is generally satisfied by the media contained within the system after initial seeding. The amount of time between the initial seeding and the start of the media perfusion is dependent on the density of cells in the seeding inoculum and the cell growth rate. The measurement of nutrient concentration in the circulating media is a good indicator of the status of the culture. When establishing a procedure it may be necessary to monitor the nutrients composition at a variety of different perfusion rates to determine the most economical and productive operating parameters.

25

Cells within the system reach a higher density of solution (cells/ml) than in traditional culture systems. Many typically used basal media are designed to support $1-2 \times 10^6$ cells/ml/day. A typical Cellcube™, run with an 85,000 cm² surface, contains approximately 6L media within the module. The cell density often exceeds 10^7 cells/mL in the culture vessel. At confluence, 2-4 reactor volumes of media are required per day.

30

The timing and parameters of the production phase of cultures depends on the type and use of a particular cell line. Many cultures require a different media for production than is required for the growth phase of the culture. The transition from one phase to the other will likely require multiple washing steps in traditional cultures. However, the Cellcube™ system employs a perfusion system. On of the benefits of such a system is the ability to provide a gentle transition between various operating phases. The perfusion system negates the need for traditional wash steps that seek to remove serum components in a growth medium.

In an exemplary embodiment of the present invention, the CellCube™ system is used to grow cells transfected with AdCMVp53. 293 cells were inoculated into the Cellcube™ according to the manufacturer's recommendation. Inoculation cell densities were in the range of $1-1.5 \times 10^4/\text{cm}^2$. Cells were allowed to grow for 7 days at 37°C under culture conditions of pH=7.20, DO=60% air saturation. The medium perfusion rate was regulated according to the glucose concentration in the Cellcube™. One day before viral infection, medium for perfusion was changed from a buffer comprising 10% FBS to a buffer comprising 2% FBS. On day 8, cells were infected with virus at a multiplicity of infection (MOI) of 5. Medium perfusion was stopped for 1 hr immediately after infection then resumed for the remaining period of the virus production phase. Culture was harvested 45-48 hr post-infection. Of course these culture conditions are exemplary and may be varied according to the nutritional needs and growth requirements of a particular cell line. Such variation may be performed without undue experimentation and are well within the skill of the ordinary person in the art.

G) Serum-Free Suspension Culture

In particular embodiments, adenoviral vectors for gene therapy are produced from anchorage-dependent culture of 293 cells (293A cells) as described above. Scale-up of adenoviral vector production is constrained by the anchorage-dependency of 293A cells. To facilitate scale-up and meet future demand for adenoviral vectors,

significant efforts have been devoted to the development of alternative production processes that are amenable to scale-up. Methods include growing 293A cells in microcarrier cultures and adaptation of 293A producer cells into suspension cultures. Microcarrier culture techniques have been described above. This technique relies on
5 the attachment of producer cells onto the surfaces of microcarriers which are suspended in culture media by mechanical agitation. The requirement of cell attachment may present some limitations to the scaleability of microcarrier cultures.

Until the present application there have been no reports on the use of 293
10 suspension cells for adenoviral vector production for gene therapy. Furthermore, the reported suspension 293 cells require the presence of 5-10% FBS in the culture media for optimal cell growth and virus production. Historically, presence of bovine source proteins in cell culture media has been a regulatory concerns, especially recently because of the outbreak of Bovine Spongiform Encephalopathy (BSE) in some
15 countries. Rigorous and complex downstream purification process has to be developed to remove contaminating proteins and any adventitious viruses from the final product. Development of serum-free 293 suspension culture is deemed to be a major process improvement for the production of adenoviral vector for gene therapy.

20 Results of virus production in spinner flasks and a 3 L stirred tank bioreactor indicate that cell specific virus productivity of the 293SF cells was approximately 2.5×10^4 vp/cell, which is approximately 60-90% of that from the 293A cells. However, because of the higher stationary cell concentration, volumetric virus productivity from the 293SF culture is essentially equivalent to that of the 293A cell
25 culture. The inventors also observed that virus production increased significantly by carrying out a fresh medium exchange at the time of virus infection. The inventors are going to evaluate the limiting factors in the medium.

These findings allow for a scaleable, efficient, and easily validatable process
30 for the production adenoviral vector. This adaptation method is not limited to 293A

cells only and will be equally useful when applied to other adenoviral vector producer cells.

3. Methods of Cell Harvest and Lysis

5 Adenoviral infection results in the lysis of the cells being infected. The lytic characteristics of adenovirus infection permit two different modes of virus production. One is harvesting infected cells prior to cell lysis. The other mode is harvesting virus supernatant after complete cell lysis by the produced virus. For the latter mode, longer incubation times are required in order to achieve complete cell lysis. This
10 prolonged incubation time after virus infection creates a serious concern about increased possibility of generation of replication competent adenovirus (RCA), particularly for the current first generation adenoviral vectors (E1-deleted vector). Therefore, harvesting infected cells before cell lysis was chosen as the production mode of choice. Table 1 lists the most common methods that have been used for
15 lysing cells after cell harvest.

TABLE 1. Methods used for cell lysis

Methods	Procedures	Comments
Freeze-thaw	Cycling between dry ice and 37°C water bath	Easy to carry out at lab scale. High cell lysis efficiency Not scaleable Not recommended for large scale manufacturing
Solid Shear	French Press Hughes Press	Capital equipment investment Virus containment concerns Lack of experience
Detergent lysis	Non-ionic detergent solutions such as Tween, Triton, NP-40, etc.	Easy to carry out at both lab and manufacturing scale Wide variety of detergent choices Concerns of residual detergent in finished product
Hypotonic solution lysis	water, citric buffer	Low lysis efficiency
Liquid Shear	Homogenizer Impinging Jet Microfluidizer	Capital equipment investment Virus containment concerns Scaleability concerns
Sonication	ultrasound	Capital equipment investment Virus containment concerns Noise pollution Scaleability concern

A) Detergents

- 5 Cells are bounded by membranes. In order to release components of the cell, it is necessary to break open the cells. The most advantageous way in which this can be accomplished, according to the present invention, is to solubilize the membranes with the use of detergents. Detergents are amphipathic molecules with an apolar end of aliphatic or aromatic nature and a polar end which may be charged or uncharged.
- 10 Detergents are more hydrophilic than lipids and thus have greater water solubility than lipids. They allow for the dispersion of water insoluble compounds into aqueous media and are used to isolate and purify proteins in a native form.

Detergents can be denaturing or non-denaturing. The former can be anionic such as sodium dodecyl sulfate or cationic such as ethyl trimethyl ammonium bromide. These detergents totally disrupt membranes and denature the protein by
5 breaking protein-protein interactions. Non denaturing detergents can be divided into non-anionic detergents such as Triton®X-100, bile salts such as cholates and zwitterionic detergents such as CHAPS. Zwitterionics contain both cationic and anion groups in the same molecule, the positive electric charge is neutralized by the negative charge on the same or adjacent molecule.

10

Denaturing agents such as SDS bind to proteins as monomers and the reaction is equilibrium driven until saturated. Thus, the free concentration of monomers determines the necessary detergent concentration. SDS binding is cooperative i.e. the binding of one molecule of SDS increase the probability of another molecule binding
15 to that protein, and alters proteins into rods whose length is proportional to their molecular weight.

Non-denaturing agents such as Triton®X-100 do not bind to native conformations nor do they have a cooperative binding mechanism. These detergents
20 have rigid and bulky apolar moieties that do not penetrate into water soluble proteins. They bind to the hydrophobic parts of proteins. Triton®X100 and other polyoxyethylene nonanionic detergents are inefficient in breaking protein-protein interaction and can cause artifactual aggregations of protein. These detergents will, however, disrupt protein-lipid interactions but are much gentler and capable of
25 maintaining the native form and functional capabilities of the proteins.

Detergent removal can be attempted in a number of ways. Dialysis works well with detergents that exist as monomers. Dialysis is somewhat ineffective with detergents that readily aggregate to form micelles because they micelles are too large
30 to pass through dialysis. Ion exchange chromatography can be utilized to circumvent this problem. The disrupted protein solution is applied to an ion exchange

chromatography column and the column is then washed with buffer minus detergent. The detergent will be removed as a result of the equilibration of the buffer with the detergent solution. Alternatively the protein solution may be passed through a density gradient. As the protein sediments through the gradients the detergent will come off
5 due to the chemical potential.

Often a single detergent is not versatile enough for the solubilization and analysis of the milieu of proteins found in a cell. The proteins can be solubilized in one detergent and then placed in another suitable detergent for protein analysis. The
10 protein detergent micelles formed in the first step should separate from pure detergent micelles. When these are added to an excess of the detergent for analysis, the protein is found in micelles with both detergents. Separation of the detergent-protein micelles can be accomplished with ion exchange or gel filtration chromatography, dialysis or buoyant density type separations.

15

Triton®X- Detergents: This family of detergents (Triton®X-100, X114 and NP-40) have the same basic characteristics but are different in their specific hydrophobic-hydrophilic nature. All of these heterogeneous detergents have a branched 8-carbon chain attached to an aromatic ring. This portion of the molecule
20 contributes most of the hydrophobic nature of the detergent. Triton®X detergents are used to solubilize membrane proteins under non-denaturing conditions. The choice of detergent to solubilize proteins will depend on the hydrophobic nature of the protein to be solubilized. Hydrophobic proteins require hydrophobic detergents to effectively solubilize them.

25

Triton®X-100 and NP-40 are very similar in structure and hydrophobicity and are interchangeable in most applications including cell lysis, delipidation protein dissociation and membrane protein and lipid solubilization. Generally 2 mg detergent is used to solubilize 1mg membrane protein or 10mg detergent/1mg of lipid
30 membrane. Triton®X-114 is useful for separating hydrophobic from hydrophilic proteins.

Brij[®] Detergents: These are similar in structure to Triton[®]X detergents in that they have varying lengths of polyoxyethylene chains attached to a hydrophobic chain. However, unlike Triton[®]X detergents, the Brij[®] detergents do not have an aromatic ring and the length of the carbon chains can vary. The Brij[®] detergents are difficult to remove from solution using dialysis but may be removed by detergent removing gels. Brij[®]58 is most similar to Triton[®]X100 in its hydrophobic/hydrophilic characteristics. Brij[®]-35 is a commonly used detergent in HPLC applications.

Dializable Nonionic Detergents: η -Octyl- β -D-glucoside (octylglucopyranoside) and η -Octyl- β -D-thioglucoside (octylthioglucopyranoside, OTG) are nondenaturing nonionic detergents which are easily dialyzed from solution. These detergents are useful for solubilizing membrane proteins and have low UV absorbances at 280 nm. Octylglucoside has a high CMC of 23-25 mM and has been used at concentrations of 1.1-1.2% to solubilize membrane proteins.

Octylthioglucoside was first synthesized to offer an alternative to octylglucoside. Octylglucoside is expensive to manufacture and there are some inherent problems in biological systems because it can be hydrolyzed by β -glucosidase.

Tween[®] Detergents: The Tween[®] detergents are nondenaturing, nonionic detergents. They are polyoxyethylene sorbitan esters of fatty acids. Tween[®] 20 and Tween[®] 80 detergents are used as blocking agents in biochemical applications and are usually added to protein solutions to prevent nonspecific binding to hydrophobic materials such as plastics or nitrocellulose. They have been used as blocking agents in ELISA and blotting applications. Generally, these detergents are used at concentrations of 0.01-1.0% to prevent nonspecific binding to hydrophobic materials.

Tween[®] 20 and other nonionic detergents have been shown to remove some proteins from the surface of nitrocellulose. Tween[®] 80 has been used to solubilize

membrane proteins, present nonspecific binding of protein to multiwell plastic tissue culture plates and to reduce nonspecific binding by serum proteins and biotinylated protein A to polystyrene plates in ELISA.

5 The difference between these detergents is the length of the fatty acid chain. Tween[®] 80 is derived from oleic acid with a C₁₈ chain while Tween[®] 20 is derived from lauric acid with a C₁₂ chain. The longer fatty acid chain makes the Tween[®] 80 detergent less hydrophilic than Tween[®] 20 detergent. Both detergents are very soluble in water.

10

The Tween[®] detergents are difficult to remove from solution by dialysis, but Tween[®] 20 can be removed by detergent removing gels. The polyoxyethylene chain found in these detergents makes them subject to oxidation (peroxide formation) as is true with the Triton[®] X and Brij[®] series detergents.

15

Zwitterionic Detergents: The zwitterionic detergent, CHAPS, is a sulfobetaine derivative of cholic acid. This zwitterionic detergent is useful for membrane protein solubilization when protein activity is important. This detergent is useful over a wide range of pH (pH 2-12) and is easily removed from solution by
20 dialysis due to high CMCs (8-10 mM). This detergent has low absorbances at 280 nm making it useful when protein monitoring at this wavelength is necessary. CHAPS is compatible with the BCA Protein Assay and can be removed from solution by detergent removing gel. Proteins can be iodinated in the presence of CHAPS.

25 CHAPS has been successfully used to solubilize intrinsic membrane proteins and receptors and maintain the functional capability of the protein. When cytochrome P-450 is solubilized in either Triton[®] X-100 or sodium cholate aggregates are formed.

B) Non-Detergent Methods

30 Various non-detergent methods, though not preferred, may be employed in conjunction with other advantageous aspects of the present invention:

Freeze-Thaw: This has been a widely used technique for lysis cells in a gentle and effective manner. Cells are generally frozen rapidly in, for example, a dry ice/ethanol bath until completely frozen, then transferred to a 37°C bath until
5 completely thawed. This cycle is repeated a number of times to achieve complete cell lysis.

Sonication: High frequency ultrasonic oscillations have been found to be useful for cell disruption. The method by which ultrasonic waves break cells is not
10 fully understood but it is known that high transient pressures are produced when suspensions are subjected to ultrasonic vibration. The main disadvantage with this technique is that considerable amounts of heat are generated. In order to minimize heat effects specifically designed glass vessels are used to hold the cell suspension. Such designs allow the suspension to circulate away from the ultrasonic probe to the
15 outside of the vessel where it is cooled as the flask is suspended in ice.

High Pressure Extrusion: This is a frequently used method to disrupt microbial cell. The French pressure cell employs pressures of 10.4×10^7 Pa (16,000 p.s.i) to break cells open. These apparatus consists of a stainless steel chamber which
20 opens to the outside by means of a needle valve. The cell suspension is placed in the chamber with the needle valve in the closed position. After inverting the chamber, the valve is opened and the piston pushed in to force out any air in the chamber. With the valve in the closed position, the chamber is restored to its original position, placed on a solid based and the required pressure is exerted on the piston by a hydraulic press.
25 When the pressure has been attained the needle valve is opened fractionally to slightly release the pressure and as the cells expand they burst. The valve is kept open while the pressure is maintained so that there is a trickle of ruptured cell which may be collected.

30 **Solid Shear Methods:** Mechanical shearing with abrasives may be achieved in Mickle shakers which oscillate suspension vigorously (300-3000

time/min) in the presence of glass beads of 500nm diameter. This method may result in organelle damage. A more controlled method is to use a Hughes press where a piston forces most cells together with abrasives or deep frozen paste of cells through a 0.25mm diameter slot in the pressure chamber. Pressures of up to 5.5×10^7 Pa (8000 p.s.i.) may be used to lyse bacterial preparations.

Liquid Shear Methods: These methods employ blenders, which use high speed reciprocating or rotating blades, homogenizers which use an upward/downward motion of a plunger and ball and microfluidizers or impinging jets which use high velocity passage through small diameter tubes or high velocity impingement of two fluid streams. The blades of blenders are inclined at different angles to permit efficient mixing. Homogenizers are usually operated in short high speed bursts of a few seconds to minimize local heat. These techniques are not generally suitable for microbial cells but even very gentle liquid shear is usually adequate to disrupt animal cells.

Hypotonic/Hypertonic Methods: Cells are exposed to a solution with a much lower (hypotonic) or higher (hypertonic) solute concentration. The difference in solute concentration creates an osmotic pressure gradient. The resulting flow of water into the cell in a hypotonic environment causes the cells to swell and burst. The flow of water out of the cell in a hypertonic environment causes the cells to shrink and subsequently burst.

4. Methods of Concentration and Filtration

One aspect of the present invention employs methods of crude purification of adenovirus from a cell lysate. These methods include clarification, concentration and diafiltration. The initial step in this purification process is clarification of the cell lysate to remove large particulate matter, particularly cellular components, from the cell lysate. Clarification of the lysate can be achieved using a depth filter or by tangential flow filtration. In a preferred embodiment of the present invention, the cell lysate is passed through a depth filter, which consists of a packed column of relatively

non-adsorbent material (*e.g.* polyester resins, sand, diatomeaceous earth, colloids, gels, and the like). In tangential flow filtration (TFF), the lysate solution flows across a membrane surface which facilitates back diffusion of solute from the membrane surface into the bulk solution. Membranes are generally arranged within various types of filter apparatus including open channel plate and frame, hollow fibers, and tubules.

After clarification and prefiltration of the cell lysate, the resultant virus supernatant is first concentrated and then the buffer is exchanged by diafiltration. The virus supernatant is concentrated by tangential flow filtration across an ultrafiltration membrane of 100-300K nominal molecular weight cutoff. Ultrafiltration is a pressure-modified convective process that uses semi-permeable membranes to separate species by molecular size, shape and/or charge. It separates solvents from solutes of various sizes, independent of solute molecular size. Ultrafiltration is gentle, efficient and can be used to simultaneously concentrate and desalt solutions. Ultrafiltration membranes generally have two distinct layers: a thin (0.1-1.5 μm), dense skin with a pore diameter of 10-400 angstroms and an open substructure of progressively larger voids which are largely open to the permeate side of the ultrafilter. Any species capable of passing through the pores of the skin can therefore freely pass through the membrane. For maximum retention of solute, a membrane is selected that has a nominal molecular weight cut-off well below that of the species being retained. In macromolecular concentration, the membrane enriches the content of the desired biological species and provides filtrate cleared of retained substances. Microsolutes are removed convectively with the solvent. As concentration of the retained solute increases, the ultrafiltration rate diminishes.

Diafiltration, or buffer exchange, using ultrafilters is an ideal way for removal and exchange of salts, sugars, non-aqueous solvents separation of free from bound species, removal of material of low molecular weight, or rapid change of ionic and pH environments. Microsolutes are removed most efficiently by adding solvent to the solution being ultrafiltered at a rate equal to the ultrafiltration rate. This washes

microspecies from the solution at constant volume, purifying the retained species. The present invention utilizes a diafiltration step to exchange the buffer of the virus supernatant prior to Benzonase[®] treatment.

5 5. **Viral Infection**

The present invention employs, in one example, adenoviral infection of cells in order to generate therapeutically significant vectors. Typically, the virus will simply be exposed to the appropriate host cell under physiologic conditions, permitting uptake of the virus. Though adenovirus is exemplified, the present
10 methods may be advantageously employed with other viral vectors, as discussed below.

A) Adenovirus

Adenovirus is particularly suitable for use as a gene transfer vector because of
15 its mid-sized DNA genome, ease of manipulation, high titer, wide target-cell range, and high infectivity. The roughly 36 kB viral genome is bounded by 100-200 base pair (bp) inverted terminal repeats (ITR), in which are contained *cis*-acting elements necessary for viral DNA replication and packaging. The early (E) and late (L) regions of the genome that contain different transcription units are divided by the onset of
20 viral DNA replication.

The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B) results in the synthesis of the proteins for viral DNA
25 replication. These proteins are involved in DNA replication, late gene expression, and host cell shut off (Renan, 1990). The products of the late genes (L1, L2, L3, L4 and L5), including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at 16.8 map units) is particularly efficient during the late
30 phase of infection, and all the mRNAs issued from this promoter possess a 5' tripartite leader (TL) sequence which makes them preferred mRNAs for translation.

In order for adenovirus to be optimized for gene therapy, it is necessary to maximize the carrying capacity so that large segments of DNA can be included. It also is very desirable to reduce the toxicity and immunologic reaction associated with certain adenoviral products. Elimination of large portions of the adenoviral genome, and providing the delete gene products *in trans*, by helper virus and/or helper cells, allows for the insertion of large portions of heterologous DNA into the vector. This strategy also will result in reduced toxicity and immunogenicity of the adenovirus gene products.

10

The large displacement of DNA is possible because the *cis* elements required for viral DNA replication all are localized in the inverted terminal repeats (ITR) (100-200 bp) at either end of the linear viral genome. Plasmids containing ITR's can replicate in the presence of a non-defective adenovirus (Hay *et al.*, 1984). Therefore, inclusion of these elements in an adenoviral vector should permit replication.

In addition, the packaging signal for viral encapsidation is localized between 194-385 bp (0.5-1.1 map units) at the left end of the viral genome (Hearing *et al.*, 1987). This signal mimics the protein recognition site in bacteriophage λ DNA where a specific sequence close to the left end, but outside the cohesive end sequence, mediates the binding to proteins that are required for insertion of the DNA into the head structure. E1 substitution vectors of Ad have demonstrated that a 450 bp (0-1.25 map units) fragment at the left end of the viral genome could direct packaging in 293 cells (Levrero *et al.*, 1991).

25

Previously, it has been shown that certain regions of the adenoviral genome can be incorporated into the genome of mammalian cells and the genes encoded thereby expressed. These cell lines are capable of supporting the replication of an adenoviral vector that is deficient in the adenoviral function encoded by the cell line. There also have been reports of complementation of replication deficient adenoviral vectors by "helping" vectors, *e.g.*, wild-type virus or conditionally defective mutants.

30

Replication-deficient adenoviral vectors can be complemented, in *trans*, by helper virus. This observation alone does not permit isolation of the replication-deficient vectors, however, since the presence of helper virus, needed to provide replicative functions, would contaminate any preparation. Thus, an additional element was needed that would add specificity to the replication and/or packaging of the replication-deficient vector. That element, as provided for in the present invention, derives from the packaging function of adenovirus.

It has been shown that a packaging signal for adenovirus exists in the left end of the conventional adenovirus map (Tibbetts, 1977). Later studies showed that a mutant with a deletion in the E1A (194-358 bp) region of the genome grew poorly even in a cell line that complemented the early (E1A) function (Hearing and Shenk, 1983). When a compensating adenoviral DNA (0-353 bp) was recombined into the right end of the mutant, the virus was packaged normally. Further mutational analysis identified a short, repeated, position-dependent element in the left end of the Ad5 genome. One copy of the repeat was found to be sufficient for efficient packaging if present at either end of the genome, but not when moved towards the interior of the Ad5 DNA molecule (Hearing *et al.*, 1987).

By using mutated versions of the packaging signal, it is possible to create helper viruses that are packaged with varying efficiencies. Typically, the mutations are point mutations or deletions. When helper viruses with low efficiency packaging are grown in helper cells, the virus is packaged, albeit at reduced rates compared to wild-type virus, thereby permitting propagation of the helper. When these helper viruses are grown in cells along with virus that contains wild-type packaging signals, however, the wild-type packaging signals are recognized preferentially over the mutated versions. Given a limiting amount of packaging factor, the virus containing the wild-type signals are packaged selectively when compared to the helpers. If the preference is great enough, stocks approaching homogeneity should be achieved.

B) Retrovirus

Although adenoviral infection of cells for the generation of therapeutically significant vectors is a preferred embodiment of the present invention, it is contemplated that the present invention may employ retroviral infection of cells for the purposes of generating such vectors. The retroviruses are a group of single-stranded RNA viruses characterized by an ability to convert their RNA to double-stranded DNA in infected cells by a process of reverse-transcription (Coffin, 1990). The resulting DNA then stably integrates into cellular chromosomes as a provirus and directs synthesis of viral proteins. The integration results in the retention of the viral gene sequences in the recipient cell and its descendants. The retroviral genome contains three genes - gag, pol and env - that code for capsid proteins, polymerase enzyme, and envelope components, respectively. A sequence found upstream from the gag gene, termed Y, functions as a signal for packaging of the genome into virions. Two long terminal repeat (LTR) sequences are present at the 5' and 3' ends of the viral genome. These contain strong promoter and enhancer sequences and are also required for integration in the host cell genome (Coffin, 1990).

In order to construct a retroviral vector, a nucleic acid encoding a promoter is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. In order to produce virions, a packaging cell line containing the gag, pol and env genes but without the LTR and Y components is constructed (Mann *et al.*, 1983). When a recombinant plasmid containing a human cDNA, together with the retroviral LTR and Y sequences is introduced into this cell line (by calcium phosphate precipitation for example), the Y sequence allows the RNA transcript of the recombinant plasmid to be packaged into viral particles, which are then secreted into the culture media (Nicolas and Rubenstein, 1988; Temin, 1986; Mann *et al.*, 1983). The media containing the recombinant retroviruses is then collected, optionally concentrated, and used for gene transfer. Retroviral vectors are able to infect a broad variety of cell types. However, integration and stable expression require the division of host cells (Paskind *et al.*, 1975).

A novel approach designed to allow specific targeting of retrovirus vectors was recently developed based on the chemical modification of a retrovirus by the chemical addition of galactose residues to the viral envelope. This modification could permit the specific infection of cells such as hepatocytes via asialoglycoprotein receptors, should this be desired.

A different approach to targeting of recombinant retroviruses was designed in which biotinylated antibodies against a retroviral envelope protein and against a specific cell receptor were used. The antibodies were coupled via the biotin components by using streptavidin (Roux *et al.*, 1989). Using antibodies against major histocompatibility complex class I and class II antigens, the infection of a variety of human cells that bore those surface antigens was demonstrated with an ecotropic virus in vitro (Roux *et al.*, 1989).

15 C) Other Viral Vectors

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as vaccinia virus (Ridgeway, 1988; Baichwal and Sugden, 1986; Coupar *et al.*, 1988), adeno-associated virus (AAV) (Ridgeway, 1988; Baichwal and Sugden, 1986; Hermonat and Muzycska, 1984) and herpesviruses may be employed. These viruses offer several features for use in gene transfer into various mammalian cells.

6. Engineering of Viral Vectors

In certain embodiments, the present invention further involves the manipulation of viral vectors. Such methods involve the use of a vector construct containing, for example, a heterologous DNA encoding a gene of interest and a means for its expression, replicating the vector in an appropriate helper cell, obtaining viral particles produced therefrom, and infecting cells with the recombinant virus particles. The gene could simply encode a protein for which large quantities of the protein are desired, *i.e.*, large scale *in vitro* production methods. Alternatively, the gene could be a therapeutic gene, for example to treat cancer cells, to express immunomodulatory

genes to fight viral infections, or to replace a gene's function as a result of a genetic defect. In the context of the gene therapy vector, the gene will be a heterologous DNA, meant to include DNA derived from a source other than the viral genome which provides the backbone of the vector. Finally, the virus may act as a live viral vaccine and express an antigen of interest for the production of antibodies thereagainst. The gene may be derived from a prokaryotic or eukaryotic source such as a bacterium, a virus, a yeast, a parasite, a plant, or even an animal. The heterologous DNA also may be derived from more than one source, *i.e.*, a multigene construct or a fusion protein. The heterologous DNA may also include a regulatory sequence which may be derived from one source and the gene from a different source.

A) *Therapeutic Genes*

p53 currently is recognized as a tumor suppressor gene (Montenarh, 1992). High levels of mutant p53 have been found in many cells transformed by chemical carcinogenesis, ultraviolet radiation, and several viruses, including SV40. The p53 gene is a frequent target of mutational inactivation in a wide variety of human tumors and is already documented to be the most frequently-mutated gene in common human cancers (Mercer, 1992). It is mutated in over 50% of human NSCLC (Hollestein *et al.*, 1991) and in a wide spectrum of other tumors.

The p53 gene encodes a 393-amino-acid phosphoprotein that can form complexes with host proteins such as large-T antigen and E1B. The protein is found in normal tissues and cells, but at concentrations which are generally minute by comparison with transformed cells or tumor tissue. Interestingly, wild-type p53 appears to be important in regulating cell growth and division. Overexpression of wild-type p53 has been shown in some cases to be anti-proliferative in human tumor cell lines. Thus, p53 can act as a negative regulator of cell growth (Weinberg, 1991) and may directly suppress uncontrolled cell growth or directly or indirectly activate genes that suppress this growth. Thus, absence or inactivation of wild-type p53 may contribute to transformation. However, some studies indicate that the presence of

mutant p53 may be necessary for full expression of the transforming potential of the gene.

Wild-type p53 is recognized as an important growth regulator in many cell types. Missense mutations are common for the p53 gene and are known to occur in at least 30 distinct codons, often creating dominant alleles that produce shifts in cell phenotype without a reduction to homozygosity. Additionally, many of these dominant negative alleles appear to be tolerated in the organism and passed on in the germ line. Various mutant alleles appear to range from minimally dysfunctional to strongly penetrant, dominant negative alleles (Weinberg, 1991).

Casey and colleagues have reported that transfection of DNA encoding wild-type p53 into two human breast cancer cell lines restores growth suppression control in such cells (Casey *et al.*, 1991). A similar effect has also been demonstrated on transfection of wild-type, but not mutant, p53 into human lung cancer cell lines (Takahashi *et al.*, 1992). p53 appears dominant over the mutant gene and will select against proliferation when transfected into cells with the mutant gene. Normal expression of the transfected p53 is not detrimental to normal cells with endogenous wild-type p53. Thus, such constructs might be taken up by normal cells without adverse effects. It is thus proposed that the treatment of p53-associated cancers with wild-type p53 expression constructs will reduce the number of malignant cells or their growth rate. Furthermore, recent studies suggest that some p53 wild-type tumors are also sensitive to the effects of exogenous p53 expression.

The major transitions of the eukaryotic cell cycle are triggered by cyclin-dependent kinases, or CDK's. One CDK, cyclin-dependent kinase 4 (CDK4), regulates progression through the G₁ phase. The activity of this enzyme may be to phosphorylate Rb at late G₁. The activity of CDK4 is controlled by an activating subunit, D-type cyclin, and by an inhibitory subunit, *e.g.* p16^{INK4}, which has been biochemically characterized as a protein that specifically binds to and inhibits CDK4, and thus may regulate Rb phosphorylation (Serrano *et al.*, 1993; Serrano *et al.*, 1995).

Since the p16^{INK4} protein is a CDK4 inhibitor (Serrano, 1993), deletion of this gene may increase the activity of CDK4, resulting in hyperphosphorylation of the Rb protein. p16 also is known to regulate the function of CDK6.

5 p16^{INK4} belongs to a newly described class of CDK-inhibitory proteins that also includes p16^B, p21^{WAF1, CIP1, SDI1}, and p27^{KIP1}. The p16^{INK4} gene maps to 9p21, a chromosome region frequently deleted in many tumor types. Homozygous deletions and mutations of the p16^{INK4} gene are frequent in human tumor cell lines. This evidence suggests that the p16^{INK4} gene is a tumor suppressor gene. This
10 interpretation has been challenged, however, by the observation that the frequency of the p16^{INK4} gene alterations is much lower in primary uncultured tumors than in cultured cell lines (Caldas *et al.*, 1994; Cheng *et al.*, 1994; Hussussian *et al.*, 1994; Kamb *et al.*, 1994a; Kamb *et al.*, 1994b; Mori *et al.*, 1994; Okamoto *et al.*, 1994; Nobori *et al.*, 1995; Orlow *et al.*, 1994; Arap *et al.*, 1995). Restoration of wild-type
15 p16^{INK4} function by transfection with a plasmid expression vector reduced colony formation by some human cancer cell lines (Okamoto, 1994; Arap, 1995).

C-CAM is expressed in virtually all epithelial cells (Odin and Obrink, 1987). C-CAM, with an apparent molecular weight of 105 kD, was originally isolated from
20 the plasma membrane of the rat hepatocyte by its reaction with specific antibodies that neutralize cell aggregation (Obrink, 1991). Recent studies indicate that, structurally, C-CAM belongs to the immunoglobulin (Ig) superfamily and its sequence is highly homologous to carcinoembryonic antigen (CEA) (Lin and Guidotti, 1989). Using a baculovirus expression system, Cheung *et al.* (1993a; 1993b and 1993c) demonstrated
25 that the first Ig domain of C-CAM is critical for cell adhesion activity.

Cell adhesion molecules, or CAMs are known to be involved in a complex network of molecular interactions that regulate organ development and cell differentiation (Edelman, 1985). Recent data indicate that aberrant expression of
30 CAMs may be involved in the tumorigenesis of several neoplasms; for example, decreased expression of E-cadherin, which is predominantly expressed in epithelial

cells, is associated with the progression of several kinds of neoplasms (Edelman and Crossin, 1991; Frixen *et al.*, 1991; Bussemakers *et al.*, 1992; Matura *et al.*, 1992; Umbas *et al.*, 1992). Also, Giancotti and Ruoslahti (1990) demonstrated that increasing expression of $\alpha_5\beta_1$ integrin by gene transfer can reduce tumorigenicity of Chinese hamster ovary cells *in vivo*. C-CAM now has been shown to suppress tumor growth *in vitro* and *in vivo*.

Other tumor suppressors that may be employed according to the present invention include RB, APC, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, *zac1*, p73, BRCA1, VHL, FCC, MMAC1, MCC, p16, p21, p57, C-CAM, p27 and BRCA2. Inducers of apoptosis, such as Bax, Bak, Bcl-X_s, Bik, Bid, Harakiri, Ad E1B, Bad and ICE-CED3 proteases, similarly could find use according to the present invention.

Various enzyme genes are of interest according to the present invention. Such enzymes include cytosine deaminase, hypoxanthine-guanine phosphoribosyltransferase, galactose-1-phosphate uridylyltransferase, phenylalanine hydroxylase, glucocerebrosidase, sphingomyelinase, α -L-iduronidase, glucose-6-phosphate dehydrogenase, HSV thymidine kinase and human thymidine kinase.

Hormones are another group of gene that may be used in the vectors described herein. Included are growth hormone, prolactin, placental lactogen, luteinizing hormone, follicle-stimulating hormone, chorionic gonadotropin, thyroid-stimulating hormone, leptin, adrenocorticotropin (ACTH), angiotensin I and II, β -endorphin, β -melanocyte stimulating hormone (β -MSH), cholecystokinin, endothelin I, galanin, gastric inhibitory peptide (GIP), glucagon, insulin, lipotropins, neurophysins, somatostatin, calcitonin, calcitonin gene related peptide (CGRP), β -calcitonin gene related peptide, hypercalcemia of malignancy factor (1-40), parathyroid hormone-related protein (107-139) (PTH-rP), parathyroid hormone-related protein (107-111) (PTH-rP), glucagon-like peptide (GLP-1), pancreastatin, pancreatic peptide, peptide YY, PHM, secretin, vasoactive intestinal peptide (VIP), oxytocin, vasopressin (AVP), vasotocin, enkephalinamide, metorphinamide, alpha melanocyte stimulating hormone

(alpha-MSH), atrial natriuretic factor (5-28) (ANF), amylin, amyloid P component (SAP-1), corticotropin releasing hormone (CRH), growth hormone releasing factor (GHRH), luteinizing hormone-releasing hormone (LHRH), neuropeptide Y, substance K (neurokinin A), substance P and thyrotropin releasing hormone (TRH).

5

Other classes of genes that are contemplated to be inserted into the vectors of the present invention include interleukins and cytokines. Interleukin 1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, GM-CSF and G-CSF.

10 Examples of diseases for which the present viral vector would be useful include, but are not limited to, adenosine deaminase deficiency, human blood clotting factor IX deficiency in hemophilia B, and cystic fibrosis, which would involve the replacement of the cystic fibrosis transmembrane receptor gene. The vectors embodied in the present invention could also be used for treatment of
15 hyperproliferative disorders such as rheumatoid arthritis or restenosis by transfer of genes encoding angiogenesis inhibitors or cell cycle inhibitors. Transfer of prodrug activators such as the HSV-TK gene can be also be used in the treatment of hyperploiferative disorders, including cancer.

20 **B) Antisense constructs**

Oncogenes such as *ras*, *myc*, *neu*, *raf*, *erb*, *src*, *fms*, *jun*, *trk*, *ret*, *gsp*, *hst*, *bcl* and *abl* also are suitable targets. However, for therapeutic benefit, these oncogenes would be expressed as an antisense nucleic acid, so as to inhibit the expression of the oncogene. The term "antisense nucleic acid" is intended to refer to the
25 oligonucleotides complementary to the base sequences of oncogene-encoding DNA and RNA. Antisense oligonucleotides, when introduced into a target cell, specifically bind to their target nucleic acid and interfere with transcription, RNA processing, transport and/or translation. Targeting double-stranded (ds) DNA with oligonucleotide leads to triple-helix formation; targeting RNA will lead to double-
30 helix formation.

Antisense constructs may be designed to bind to the promoter and other control regions, exons, introns or even exon-intron boundaries of a gene. Antisense RNA constructs, or DNA encoding such antisense RNAs, may be employed to inhibit gene transcription or translation or both within a host cell, either *in vitro* or *in vivo*, such as within a host animal, including a human subject. Nucleic acid sequences comprising "complementary nucleotides" are those which are capable of base-pairing according to the standard Watson-Crick complementarity rules. That is, that the larger purines will base pair with the smaller pyrimidines to form only combinations of guanine paired with cytosine (G:C) and adenine paired with either thymine (A:T), in the case of DNA, or adenine paired with uracil (A:U) in the case of RNA.

As used herein, the terms "complementary" or "antisense sequences" mean nucleic acid sequences that are substantially complementary over their entire length and have very few base mismatches. For example, nucleic acid sequences of fifteen bases in length may be termed complementary when they have a complementary nucleotide at thirteen or fourteen positions with only single or double mismatches. Naturally, nucleic acid sequences which are "completely complementary" will be nucleic acid sequences which are entirely complementary throughout their entire length and have no base mismatches.

While all or part of the gene sequence may be employed in the context of antisense construction, statistically, any sequence 17 bases long should occur only once in the human genome and, therefore, suffice to specify a unique target sequence. Although shorter oligomers are easier to make and increase *in vivo* accessibility, numerous other factors are involved in determining the specificity of hybridization. Both binding affinity and sequence specificity of an oligonucleotide to its complementary target increases with increasing length. It is contemplated that oligonucleotides of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20 or more base pairs will be used. One can readily determine whether a given antisense nucleic acid is effective at targeting of the corresponding host cell gene simply by testing the

constructs *in vitro* to determine whether the endogenous gene's function is affected or whether the expression of related genes having complementary sequences is affected.

5 In certain embodiments, one may wish to employ antisense constructs which include other elements, for example, those which include C-5 propyne pyrimidines. Oligonucleotides which contain C-5 propyne analogues of uridine and cytidine have been shown to bind RNA with high affinity and to be potent antisense inhibitors of gene expression (Wagner *et al.*, 1993).

10 As an alternative to targeted antisense delivery, targeted ribozymes may be used. The term "ribozyme" refers to an RNA-based enzyme capable of targeting and cleaving particular base sequences in oncogene DNA and RNA. Ribozymes can either be targeted directly to cells, in the form of RNA oligo-nucleotides incorporating ribozyme sequences, or introduced into the cell as an expression construct encoding
15 the desired ribozymal RNA. Ribozymes may be used and applied in much the same way as described for antisense nucleic acids.

C) *Antigens for Vaccines*

Other therapeutics genes might include genes encoding antigens such as viral
20 antigens, bacterial antigens, fungal antigens or parasitic antigens. Viruses include picornavirus, coronavirus, togavirus, flavirviro, rhabdovirus, paramyxovirus, orthomyxovirus, bunyavirus, arenavirus, reovirus, retrovirus, papovavirus, parvovirus, herpesvirus, poxvirus, hepadnavirus, and spongiform virus. Preferred viral targets include influenza, herpes simplex virus 1 and 2, measles, small pox, polio or HIV.
25 Pathogens include trypanosomes, tapeworms, roundworms, helminths, . Also, tumor markers, such as fetal antigen or prostate specific antigen, may be targeted in this manner. Preferred examples include HIV env proteins and hepatitis B surface antigen. Administration of a vector according to the present invention for vaccination purposes would require that the vector-associated antigens be sufficiently non-
30 immunogenic to enable long term expression of the transgene, for which a strong immune response would be desired. Preferably, vaccination of an individual would

only be required infrequently, such as yearly or biennially, and provide long term immunologic protection against the infectious agent.

D) Control Regions

5 In order for the viral vector to effect expression of a transcript encoding a therapeutic gene, the polynucleotide encoding the therapeutic gene will be under the transcriptional control of a promoter and a polyadenylation signal. A "promoter" refers to a DNA sequence recognized by the synthetic machinery of the host cell, or introduced synthetic machinery, that is required to initiate the specific transcription of
10 a gene. A polyadenylation signal refers to a DNA sequence recognized by the synthetic machinery of the host cell, or introduced synthetic machinery, that is required to direct the addition of a series of nucleotides on the end of the mRNA transcript for proper processing and trafficking of the transcript out of the nucleus into the cytoplasm for translation. The phrase "under transcriptional control" means that
15 the promoter is in the correct location in relation to the polynucleotide to control RNA polymerase initiation and expression of the polynucleotide.

The term promoter will be used here to refer to a group of transcriptional control modules that are clustered around the initiation site for RNA polymerase II.
20 Much of the thinking about how promoters are organized derives from analyses of several viral promoters, including those for the HSV thymidine kinase (tk) and SV40 early transcription units. These studies, augmented by more recent work, have shown that promoters are composed of discrete functional modules, each consisting of approximately 7-20 bp of DNA, and containing one or more recognition sites for
25 transcriptional activator or repressor proteins.

At least one module in each promoter functions to position the start site for RNA synthesis. The best known example of this is the TATA box, but in some promoters lacking a TATA box, such as the promoter for the mammalian terminal
30 deoxynucleotidyl transferase gene and the promoter for the SV40 late genes, a discrete element overlying the start site itself helps to fix the place of initiation.

Additional promoter elements regulate the frequency of transcriptional initiation. Typically, these are located in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well. The spacing between promoter elements frequently is flexible, so that promoter function is preserved when elements are inverted or moved relative to one another. In the tk promoter, the spacing between promoter elements can be increased to 50 bp apart before activity begins to decline. Depending on the promoter, it appears that individual elements can function either cooperatively or independently to activate transcription.

The particular promoter that is employed to control the expression of a therapeutic gene is not believed to be critical, so long as it is capable of expressing the polynucleotide in the targeted cell. Thus, where a human cell is targeted, it is preferable to position the polynucleotide coding region adjacent to and under the control of a promoter that is capable of being expressed in a human cell. Generally speaking, such a promoter might include either a human or viral promoter. A list of promoters is provided in the Table 2.

TABLE 2

PROMOTER
Immunoglobulin Heavy Chain
Immunoglobulin Light Chain
T-Cell Receptor
HLA DQ α and DQ β
β -Interferon
Interleukin-2
Interleukin-2 Receptor
MHC Class II 5
MHC Class II HLA-DR α
β -Actin
Muscle Creatine Kinase
Prealbumin (Transthyretin)
Elastase I
Metallothionein
Collagenase
Albumin Gene
α -Fetoprotein
τ -Globin
β -Globin
c-fos
c-HA-ras
Insulin
Neural Cell Adhesion Molecule (NCAM)
α_1 -Antitrypsin
H2B (TH2B) Histone
Mouse or Type I Collagen
Glucose-Regulated Proteins (GRP94 and GRP78)

PROMOTER
Rat Growth Hormone
Human Serum Amyloid A (SAA)
Troponin I (TN I)
Platelet-Derived Growth Factor
Duchenne Muscular Dystrophy
SV40
Polyoma
Retroviruses
Papilloma Virus
Hepatitis B Virus
Human Immunodeficiency Virus
Cytomegalovirus
Gibbon Ape Leukemia Virus

The promoter further may be characterized as an inducible promoter. An inducible promoter is a promoter which is inactive or exhibits low activity except in the presence of an inducer substance. Some examples of promoters that may be included as a part of the present invention include, but are not limited to, MT II, MMTV, Colleganse, Stromelysin, SV40, Murine MX gene, α -2-Macroglobulin, MHC class I gene h-2kb, HSP70, Proliferin, Tumor Necrosis Factor, or Thyroid Stimulating Hormone α gene. The associated inducers are shown in Table 3. It is understood that any inducible promoter may be used in the practice of the present invention and that all such promoters would fall within the spirit and scope of the claimed invention.

TABLE 3

Element	Inducer
MT II	Phorbol Ester (TPA) Heavy metals
MMTV (mouse mammary tumor virus)	Glucocorticoids
β -Interferon	poly(rI)X poly(rc)
Adenovirus 5 E2	Ela
c-jun	Phorbol Ester (TPA), H ₂ O ₂
Collagenase	Phorbol Ester (TPA)
Stromelysin	Phorbol Ester (TPA), IL-1
SV40	Phorbol Ester (TPA)
Murine MX Gene	Interferon, Newcastle Disease Virus
GRP78 Gene	A23187
α -2-Macroglobulin	IL-6
Vimentin	Serum
MHC Class I Gene H-2kB	Interferon
HSP70	Ela, SV40 Large T Antigen
Proliferin	Phorbol Ester-TPA
Tumor Necrosis Factor	FMA
Thyroid Stimulating Hormone α Gene	Thyroid Hormone

5

In various embodiments, the human cytomegalovirus (CMV) immediate early gene promoter, the SV40 early promoter and the Rous sarcoma virus long terminal repeat can be used to obtain high-level expression of the polynucleotide of interest. The use of other viral or mammalian cellular or bacterial phage promoters which are

10 well-known in the art to achieve expression of polynucleotides is contemplated as

well, provided that the levels of expression are sufficient to produce a growth inhibitory effect.

By employing a promoter with well-known properties, the level and pattern of expression of a polynucleotide following transfection can be optimized. For example, selection of a promoter which is active in specific cells, such as tyrosinase (melanoma), alpha-fetoprotein and albumin (liver tumors), CC10 (lung tumor) and prostate-specific antigen (prostate tumor) will permit tissue-specific expression of the therapeutic gene.

10

Enhancers were originally detected as genetic elements that increased transcription from a promoter located at a distant position on the same molecule of DNA. This ability to act over a large distance had little precedent in classic studies of prokaryotic transcriptional regulation. Subsequent work showed that regions of DNA with enhancer activity are organized much like promoters. That is, they are composed of many individual elements, each of which binds to one or more transcriptional proteins.

15

The basic distinction between enhancers and promoters is operational. An enhancer region as a whole must be able to stimulate transcription at a distance; this need not be true of a promoter region or its component elements. On the other hand, a promoter must have one or more elements that direct initiation of RNA synthesis at a particular site and in a particular orientation, whereas enhancers lack these specificities. Promoters and enhancers are often overlapping and contiguous, often seeming to have a very similar modular organization.

20
25

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base (EPDB)) could also be used to drive expression of a particular construct. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain

30

bacteriophage promoters if the appropriate bacteriophage polymerase is provided, either as part of the delivery complex or as an additional genetic expression vector.

Where a cDNA insert is employed, one will typically desire to include a
5 polyadenylation signal to effect proper polyadenylation of the gene transcript. The nature of the polyadenylation signal is not believed to be crucial to the successful practice of the invention, and any such sequence may be employed. Such polyadenylation signals as that from SV40, bovine growth hormone, and the herpes simplex virus thymidine kinase gene have been found to function well in a number of
10 target cells.

7. Methods of Gene Transfer

In order to create the helper cell lines of the present invention, and to create recombinant adenovirus vectors for use therewith, various genetic (*i.e.* DNA)
15 constructs must be delivered to a cell. One way to achieve this is via viral transductions using infectious viral particles, for example, by transformation with an adenovirus vector of the present invention. Alternatively, retroviral or bovine papilloma virus may be employed, both of which permit permanent transformation of a host cell with a gene(s) of interest. In other situations, the nucleic acid to be
20 transferred is not infectious, *i.e.*, contained in an infectious virus particle. This genetic material must rely on non-viral methods for transfer.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These
25 include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe *et al.*, 1990) DEAE-dextran (Gopal, 1985), electroporation (Tur-Kaspa *et al.*, 1986; Potter *et al.*, 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley *et al.*, 1979), cell sonication (Fechheimer *et al.*, 1987), gene bombardment using high
30 velocity microprojectiles (Yang *et al.*, 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988).

Once the construct has been delivered into the cell the nucleic acid encoding the therapeutic gene may be positioned and expressed at different sites. In certain embodiments, the nucleic acid encoding the therapeutic gene may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (gene augmentation). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In one embodiment of the invention, the expression construct may simply consist of naked recombinant DNA or plasmids. Transfer of the construct may be performed by any of the methods mentioned above which physically or chemically permeabilize the cell membrane. This is particularly applicable for transfer *in vitro*, however, it may be applied for *in vivo* use as well. Dubensky *et al.* (1984) successfully injected polyomavirus DNA in the form of CaPO₄ precipitates into liver and spleen of adult and newborn mice demonstrating active viral replication and acute infection. Benvenisty and Neshif (1986) also demonstrated that direct intraperitoneal injection of CaPO₄ precipitated plasmids results in expression of the transfected genes. It is envisioned that DNA encoding a CAM may also be transferred in a similar manner *in vivo* and express CAM.

25

Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment. This method depends on the ability to accelerate DNA coated microprojectiles to a high velocity allowing them to pierce cell membranes and enter cells without killing them (Klein *et al.*, 1987). Several devices for accelerating small particles have been developed. One such device relies on a high voltage discharge to generate an electrical current, which

30

in turn provides the motive force (Yang *et al.*, 1990). The microprojectiles used have consisted of biologically inert substances such as tungsten or gold beads.

5 In a further embodiment of the invention, the expression construct may be entrapped in a liposome. Liposomes are vesicular structures characterized by a phospholipid bilayer membrane and an inner aqueous medium. Multilamellar liposomes have multiple lipid layers separated by aqueous medium. They form spontaneously when phospholipids are suspended in an excess of aqueous solution. The lipid components undergo self-rearrangement before the formation of closed
10 structures and entrap water and dissolved solutes between the lipid bilayers (Ghosh and Bachhawat, 1991).

Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful. Using the β -lactamase gene, Wong *et al.* (1980)
15 demonstrated the feasibility of liposome-mediated delivery and expression of foreign DNA in cultured chick embryo, HeLa, and hepatoma cells. Nicolau *et al.* (1987) accomplished successful liposome-mediated gene transfer in rats after intravenous injection. Also included are various commercial approaches involving "lipofection" technology.

20

In certain embodiments of the invention, the liposome may be complexed with a hemagglutinating virus (HVJ). This has been shown to facilitate fusion with the cell membrane and promote cell entry of liposome-encapsulated DNA (Kaneda *et al.*, 1989). In other embodiments, the liposome may be complexed or employed in
25 conjunction with nuclear nonhistone chromosomal proteins (HMG-1) (Kato *et al.*, 1991). In yet further embodiments, the liposome may be complexed or employed in conjunction with both HVJ and HMG-1. In that such expression constructs have been successfully employed in transfer and expression of nucleic acid *in vitro* and *in vivo*, then they are applicable for the present invention.

30

Other expression constructs which can be employed to deliver a nucleic acid encoding a therapeutic gene into cells are receptor-mediated delivery vehicles. These take advantage of the selective uptake of macromolecules by receptor-mediated endocytosis in almost all eukaryotic cells. Because of the cell type-specific
5 distribution of various receptors, the delivery can be highly specific (Wu and Wu, 1993).

Receptor-mediated gene targeting vehicles generally consist of two components: a cell receptor-specific ligand and a DNA-binding agent. Several
10 ligands have been used for receptor-mediated gene transfer. The most extensively characterized ligands are asialoorosomucoid (ASOR) (Wu and Wu, 1987) and transferrin (Wagner *et al.*, 1990). Recently, a synthetic neoglycoprotein, which recognizes the same receptor as ASOR, has been used as a gene delivery vehicle (Ferkol *et al.*, 1993; Perales *et al.*, 1994) and epidermal growth factor (EGF) has also
15 been used to deliver genes to squamous carcinoma cells (Myers, EPO 0273085).

In other embodiments, the delivery vehicle may comprise a ligand and a liposome. For example, Nicolau *et al.* (1987) employed lactosyl-ceramide, a galactose-terminal asialoganglioside, incorporated into liposomes and observed an
20 increase in the uptake of the insulin gene by hepatocytes. Thus, it is feasible that a nucleic acid encoding a therapeutic gene also may be specifically delivered into a cell type such as prostate, epithelial or tumor cells, by any number of receptor-ligand systems with or without liposomes. For example, the human prostate-specific antigen (Watt *et al.*, 1986) may be used as the receptor for mediated delivery of a nucleic acid
25 in prostate tissue.

8. Removing Nucleic Acid Contaminants

The present invention employs nucleases to remove contaminating nucleic acids. Exemplary nucleases include Benzonase[®], Pulmozyme[®]; or any other DNase
30 or RNase commonly used within the art.

Enzymes such as Benzonase[®] degrade nucleic acid and have no proteolytic activity. The ability of Benzonase[®] to rapidly hydrolyze nucleic acids makes the enzyme ideal for reducing cell lysate viscosity. It is well known that nucleic acids may adhere to cell derived particles such as viruses. The adhesion may interfere with separation due to agglomeration, change in size of the particle or change in particle charge, resulting in little if any product being recovered with a given purification scheme. Benzonase[®] is well suited for reducing the nucleic acid load during purification, thus eliminating the interference and improving yield.

As with all endonucleases, Benzonase[®] hydrolyzes internal phosphodiester bonds between specific nucleotides. Upon complete digestion, all free nucleic acids present in solution are reduced to oligonucleotides 2 to 4 bases in length.

9. Purification Techniques

The present invention employs a number of different purification to purify adenoviral vectors of the present invention. Such techniques include those based on sedimentation and chromatography and are described in more detail herein below.

A) Density Gradient Centrifugation

There are two methods of density gradient centrifugation, the *rate zonal technique* and the *isopycnic (equal density) technique*, and both can be used when the quantitative separation of all the components of a mixture of particles is required. They are also used for the determination of buoyant densities and for the estimation of sedimentation coefficients.

Particle separation by the rate zonal technique is based upon differences in size or sedimentation rates. The technique involves carefully layering a sample solution on top of a performed liquid density gradient, the highest density of which exceeds that of the densest particles to be separated. The sample is then centrifuged until the desired degree of separation is effected, *i.e.*, for sufficient time for the particles to travel through the gradient to form discrete zones or bands which are spaced

according to the relative velocities of the particles. Since the technique is time dependent, centrifugation must be terminated before any of the separated zones pellet at the bottom of the tube. The method has been used for the separation of enzymes, hormones, RNA-DNA hybrids, ribosomal subunits, subcellular organelles, for the
5 analysis of size distribution of samples of polysomes and for lipoprotein fractionations.

The sample is layered on top of a continuous density gradient which spans the whole range of the particle densities which are to be separated. The maximum density
10 of the gradient, therefore, must always exceed the density of the most dense particle. During centrifugation, sedimentation of the particles occurs until the buoyant density of the particle and the density of the gradient are equal (*i.e.*, where $p_p = p_m$ in equation 2.12). At this point no further sedimentation occurs, irrespective of how long centrifugation continues, because the particles are floating on a cushion of material
15 that has a density greater than their own.

Isopycnic centrifugation, in contrast to the rate zonal technique, is an equilibrium method, the particles banding to form zones each at their own characteristic buoyant density. In cases where, perhaps, not all the components in a
20 mixture of particles are required, a gradient range can be selected in which unwanted components of the mixture will sediment to the bottom of the centrifuge tube whilst the particles of interest sediment to their respective isopycnic positions. Such a technique involves a combination of both the rate zonal and isopycnic approaches.

25 Isopycnic centrifugation depends solely upon the buoyant density of the particle and not its shape or size and is independent of time. Hence soluble proteins, which have a very similar density (*e.g.*, $p = 1.3 \text{ g cm}^{-3}$ in sucrose solution), cannot usually be separated by this method, whereas subcellular organelles (*e.g.*, Golgi apparatus, $p = 1.11 \text{ g cm}^{-3}$, mitochondria, $p = 1.19 \text{ g cm}^{-3}$ and peroxisomes, $p = 1.23$
30 g cm^{-3} in sucrose solution) can be effectively separated.

As an alternative to layering the particle mixture to be separated onto a preformed gradient, the sample is initially mixed with the gradient medium to give a solution of uniform density, the gradient 'self-forming', by sedimentation equilibrium, during centrifugation. In this method (referred to as the *equilibrium isodensity method*), use is generally made of the salts of heavy metals (e.g., caesium or rubidium), sucrose, colloidal silica or Metrizamide.

The sample (e.g., DNA) is mixed homogeneously with, for example, a concentrated solution of caesium chloride. Centrifugation of the concentrated caesium chloride solution results in the sedimentation of the CsCl molecules to form a concentration gradient and hence a density gradient. The sample molecules (DNA), which were initially uniformly distributed throughout the tube now either rise or sediment until they reach a region where the solution density is equal to their own buoyant density, i.e. their isopycnic position, where they will band to form zones. This technique suffers from the disadvantage that often very long centrifugation times (e.g., 36 to 48 hours) are required to establish equilibrium. However, it is commonly used in analytical centrifugation to determine the buoyant density of a particle, the base composition of double stranded DNA and to separate linear from circular forms of DNA.

Many of the separations can be improved by increasing the density differences between the different forms of DNA by the incorporation of heavy isotopes (e.g., ^{15}N) during biosynthesis, a technique used by Leselson and Stahl to elucidate the mechanism of DNA replication in *Escherichia coli*, or by the binding of heavy metal ions or dyes such as ethidium bromide. Isopycnic gradients have also been used to separate and purify viruses and analyze human plasma lipoproteins.

B) Chromatography

In certain embodiments of the invention, it will be desirable to produce purified adenovirus. Purification techniques are well known to those of skill in the art. These techniques tend to involve the fractionation of the cellular milieu to separate the

adenovirus particles from other components of the mixture. Having separated adenoviral particles from the other components, the adenovirus may be purified using chromatographic and electrophoretic techniques to achieve complete purification. Analytical methods particularly suited to the preparation of a pure adenoviral particle of the present invention are ion-exchange chromatography, size exclusion chromatography; polyacrylamide gel electrophoresis. A particularly efficient purification method to be employed in conjunction with the present invention is HPLC.

10 Certain aspects of the present invention concern the purification, and in particular embodiments, the substantial purification, of an adenoviral particle. The term "purified" as used herein, is intended to refer to a composition, isolatable from other components, wherein the adenoviral particle is purified to any degree relative to its naturally-obtainable form. A purified adenoviral particle therefore also refers to an
15 adenoviral component, free from the environment in which it may naturally occur.

 Generally, "purified" will refer to an adenoviral particle that has been subjected to fractionation to remove various other components, and which composition substantially retains its expressed biological activity. Where the term
20 "substantially purified" is used, this designation will refer to a composition in which the particle, protein or peptide forms the major component of the composition, such as constituting about 50% or more of the constituents in the composition.

 Various methods for quantifying the degree of purification of a protein or
25 peptide will be known to those of skill in the art in light of the present disclosure. These include, for example, determining the specific activity of an active fraction, or assessing the amount of polypeptides within a fraction by SDS/PAGE analysis. A preferred method for assessing the purity of a fraction is to calculate the specific activity of the fraction, to compare it to the specific activity of the initial extract, and
30 to thus calculate the degree of purity, herein assessed by a "-fold purification number". The actual units used to represent the amount of activity will, of course, be dependent

upon the particular assay technique chosen to follow the purification and whether or not the expressed protein or peptide exhibits a detectable activity.

There is no general requirement that the adenovirus, always be provided in their most purified state. Indeed, it is contemplated that less substantially purified products will have utility in certain embodiments. Partial purification may be accomplished by using fewer purification steps in combination, or by utilizing different forms of the same general purification scheme. For example, it is appreciated that a cation-exchange column chromatography performed utilizing an HPLC apparatus will generally result in a greater -fold purification than the same technique utilizing a low pressure chromatography system. Methods exhibiting a lower degree of relative purification may have advantages in total recovery of protein product, or in maintaining the activity of an expressed protein.

Of course, it is understood that the chromatographic techniques and other purification techniques known to those of skill in the art may also be employed to purify proteins expressed by the adenoviral vectors of the present invention. Ion exchange chromatography and high performance liquid chromatography are exemplary purification techniques employed in the purification of adenoviral particles and are described in further detail herein below.

Ion-Exchange Chromatography. The basic principle of ion-exchange chromatography is that the affinity of a substance for the exchanger depends on both the electrical properties of the material and the relative affinity of other charged substances in the solvent. Hence, bound material can be eluted by changing the pH, thus altering the charge of the material, or by adding competing materials, of which salts are but one example. Because different substances have different electrical properties, the conditions for release vary with each bound molecular species. In general, to get good separation, the methods of choice are either continuous ionic strength gradient elution or stepwise elution. (A gradient of pH alone is not often used because it is difficult to set up a pH gradient without simultaneously increasing

ionic strength.) For an anion exchanger, either pH and ionic strength are gradually increased or ionic strength alone is increased. For a cation exchanger, both pH and ionic strength are increased. The actual choice of the elution procedure is usually a result of trial and error and of considerations of stability. For example, for unstable materials, it is best to maintain fairly constant pH.

An ion exchanger is a solid that has chemically bound charged groups to which ions are electrostatically bound; it can exchange these ions for ions in aqueous solution. Ion exchangers can be used in column chromatography to separate molecules according to charge; actually other features of the molecule are usually important so that the chromatographic behavior is sensitive to the charge density, charge distribution, and the size of the molecule.

The principle of ion-exchange chromatography is that charged molecules adsorb to ion exchangers reversibly so that molecules can be bound or eluted by changing the ionic environment. Separation on ion exchangers is usually accomplished in two stages: first, the substances to be separated are bound to the exchanger, using conditions that give stable and tight binding; then the column is eluted with buffers of different pH, ionic strength, or composition and the components of the buffer compete with the bound material for the binding sites.

An ion exchanger is usually a three-dimensional network or matrix that contains covalently linked charged groups. If a group is negatively charged, it will exchange positive ions and is a cation exchanger. A typical group used in cation exchangers is the sulfonic group, SO_3^- . If an H^+ is bound to the group, the exchanger is said to be in the acid form; it can, for example, exchange on H^+ for one Na^+ or two H^+ for one Ca^{2+} . The sulfonic acid group is called a strongly acidic cation exchanger. Other commonly used groups are phenolic hydroxyl and carboxyl, both weakly acidic cation exchangers. If the charged group is positive - for example, a quaternary amino group--it is a strongly basic anion exchanger. The most common weakly basic anion exchangers are aromatic or aliphatic amino groups.

The matrix can be made of various material. Commonly used materials are dextran, cellulose, agarose and copolymers of styrene and vinylbenzene in which the divinylbenzene both cross-links the polystyrene strands and contains the charged groups. Table 4 gives the composition of many ion exchangers.

The total capacity of an ion exchanger measures its ability to take up exchangeable groups per milligram of dry weight. This number is supplied by the manufacturer and is important because, if the capacity is exceeded, ions will pass through the column without binding.

TABLE 4

Matrix	Exchanger	Functional Group	Tradename
Dextran	Strong Cationic	Sulfopropyl	SP-Sephadex
	Weak Cationic	Carboxymethyl	CM-Sephadex
	Strong Anionic	Diethyl-(2-hydroxypropyl)-aminoethyl	QAE-Sephadex
	Weak Anionic	Diethylaminoethyl	DEAE-Sephadex
Cellulose	Cationic	Carboxymethyl	CM-Cellulose
	Cationic	Phospho	P-cel
	Anionic	Diethylaminoethyl	DEAE-cellulose
	Anionic	Polyethylenimine	PEI-Cellulose
	Anionic	Benzoylated-naphthoylated, diethylaminoethyl	DEAE(BND)-cellulose
	Anionic	p-Aminobenzyl	PAB-cellulose
Styrene-divinyl-benzene	Strong Cationic	Sulfonic acid	AG 50
	Strong Anionic		AG 1
	Strong Cationic	Sulfonic acid +	AG 501
	+	Tetramethylammonium	
	Strong Anionic		
Acrylic	Weak Cationic	Carboxylic	Bio-Rex 70
Phenolic	Strong Cationic	Sulfonic acid	Bio-Rex 40
Expoxyamine	Weak Anionic	Tertiary amino	AG-3

The available capacity is the capacity under particular experimental conditions (i.e., pH, ionic strength). For example, the extent to which an ion exchanger is charged depends on the pH (the effect of pH is smaller with strong ion exchangers). Another factor is ionic strength because small ions near the charged groups compete with the sample molecule for these groups. This competition is quite effective if the sample is a macromolecule because the higher diffusion coefficient of the small ion means a greater number of encounters. Clearly, as buffer concentration increases, competition becomes keener.

The porosity of the matrix is an important feature because the charged groups are both inside and outside the matrix and because the matrix also acts as a molecular sieve. Large molecules may be unable to penetrate the pores; so the capacity will decrease with increasing molecular dimensions. The porosity of the polystyrene-based resins is determined by the amount of cross-linking by the divinylbenzene (porosity decreases with increasing amounts of divinylbenzene). With the Dowex and AG series, the percentage of divinylbenzene is indicated by a number after an X - hence, Dowex 50-X8 is 8% divinylbenzene

Ion exchangers come in a variety of particle sizes, called mesh size. Finer mesh means an increased surface-to-volume ration and therefore increased capacity and decreased time for exchange to occur for a given volume of the exchanger. On the other hand, fine mesh means a slow flow rate, which can increase diffusional spreading. The use of very fine particles, approximately 10 μm in diameter and high pressure to maintain an adequate flow is called *high-performance* or *high-pressure liquid chromatography* or simply HPLC.

Such a collection of exchangers having such different properties - charge, capacity, porosity, mesh - makes the selection of the appropriate one for accomplishing a particular separation difficult. How to decide on the type of column material and the conditions for binding and elution is described in the following Examples.

There are a number of choice to be made when employing ion exchange chromatography as a technique. The first choice to be made is whether the exchanger is to be anionic or cationic. If the materials to be bound to the column have a single
5 charge (i.e., either plus or minus), the choice is clear. However, many substances (e.g., proteins, viruses), carry both negative and positive charges and the net charge depends on the pH. In such cases, the primary factor is the stability of the substance at various pH values. Most proteins have a pH range of stability (i.e., in which they do not denature) in which they are either positively or negatively charged. Hence, if a
10 protein is stable at pH values above the isoelectric point, an anion exchanger should be used; if stable at values below the isoelectric point, a cation exchanger is required.

The choice between strong and weak exchangers is also based on the effect of pH on charge and stability. For example, if a weakly ionized substance that requires
15 very low or high pH for ionization is chromatographed, a strong ion exchanger is called for because it functions over the entire pH range. However, if the substance is labile, weak ion exchangers are preferable because strong exchangers are often capable of distorting a molecule so much that the molecule denatures. The pH at which the substance is stable must, of course, be matched to the narrow range of pH in
20 which a particular weak exchanger is charged. Weak ion exchangers are also excellent for the separation of molecules with a high charge from those with a small charge, because the weakly charged ions usually fail to bind. Weak exchangers also show greater resolution of substances if charge differences are very small. If a macromolecule has a very strong charge, it may be impossible to elute from a strong
25 exchanger and a weak exchanger again may be preferable. In general, weak exchangers are more useful than strong exchangers.

The Sephadex and Bio-gel exchangers offer a particular advantage for macromolecules that are unstable in low ionic strength. Because the cross-links in
30 these materials maintain the insolubility of the matrix even if the matrix is highly polar, the density of ionizable groups can be made several times greater than is

possible with cellulose ion exchangers. The increased charge density means increased affinity so that adsorption can be carried out at higher ionic strengths. On the other hand, these exchangers retain some of their molecular sieving properties so that sometimes molecular weight differences annul the distribution caused by the charge differences; the molecular sieving effect may also enhance the separation.

Small molecules are best separated on matrices with small pore size (high degree of cross-linking) because the available capacity is large, whereas macromolecules need large pore size. However, except for the Sephadex type, most ion exchangers do not afford the opportunity for matching the porosity with the molecular weight.

The cellulose ion exchangers have proved to be the best for purifying large molecules such as proteins and polynucleotides. This is because the matrix is fibrous, and hence all functional groups are on the surface and available to even the largest molecules. In many cases however, beaded forms such as DEAE-Sephacel and DEAE-Biogel P are more useful because there is a better flow rate and the molecular sieving effect aids in separation.

Selecting a mesh size is always difficult. Small mesh size improves resolution but decreases flow rate, which increases zone spreading and decreases resolution. Hence, the appropriate mesh size is usually determined empirically.

Because buffers themselves consist of ions, they can also exchange, and the pH equilibrium can be affected. To avoid these problems, the *rule of buffers* is adopted: use *cationic buffers with anion exchangers* and *anionic buffers with cation exchangers*. Because ionic strength is a factor in binding, a buffer should be chosen that has a high buffering capacity so that its ionic strength need not be too high. Furthermore, for best resolution, it has been generally found that the ionic conditions used to apply the sample to the column (the so-called *starting conditions*) should be near those used for eluting the column.

High Performance Liquid Chromatography (HPLC) is characterized by a very rapid separation with extraordinary resolution of peaks. This is achieved by the use of very fine particles and high pressure to maintain an adequate flow rate. Separation
5 can be accomplished in a matter of minutes, or at most an hour. Moreover, only a very small volume of the sample is needed because the particles are so small and close-packed that the void volume is a very small fraction of the bed volume. Also, the concentration of the sample need not be very great because the bands are so narrow that there is very little dilution of the sample.

10

10. Pharmaceutical Compositions and Formulations

When purified according to the methods set forth above, the viral particles of the present invention will be administered, *in vitro*, *ex vivo* or *in vivo* is contemplated. Thus, it will be desirable to prepare the complex as a pharmaceutical composition
15 appropriate for the intended application. Generally this will entail preparing a pharmaceutical composition that is essentially free of pyrogens, as well as any other impurities that could be harmful to humans or animals. One also will generally desire to employ appropriate salts and buffers to render the complex stable and allow for complex uptake by target cells.

20

Aqueous compositions of the present invention comprise an effective amount of the expression construct and nucleic acid, dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. Such compositions can also be referred to as inocula. The phrases "pharmaceutically or pharmacologically
25 acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, or a human, as appropriate. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents and the like. The use of such media and
30 agents for pharmaceutical active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active ingredient, its use

in the therapeutic compositions is contemplated. Supplementary active ingredients also can be incorporated into the compositions.

5 Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions also can be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

10

The viral particles of the present invention may include classic pharmaceutical preparations for use in therapeutic regimens, including their administration to humans. Administration of therapeutic compositions according to the present invention will be via any common route so long as the target tissue is available via that route. This includes oral, nasal, buccal, rectal, vaginal or topical. Alternatively, administration will be by orthotopic, intradermal subcutaneous, intramuscular, intraperitoneal, or intravenous injection. Such compositions would normally be administered as pharmaceutically acceptable compositions that include physiologically acceptable carriers, buffers or other excipients. For application against tumors, direct intratumoral injection, inject of a resected tumor bed, regional (*i.e.*, lymphatic) or general administration is contemplated. It also may be desired to perform continuous perfusion over hours or days via a catheter to a disease site, *e.g.*, a tumor or tumor site.

25 The therapeutic compositions of the present invention are advantageously administered in the form of injectable compositions either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. These preparations also may be emulsified. A typical composition for such purpose comprises a pharmaceutically acceptable carrier. For instance, the composition may contain about 100 mg of human serum albumin per milliliter of phosphate buffered saline. Other pharmaceutically acceptable carriers include aqueous solutions, non-toxic excipients, including salts, preservatives, buffers

30

and the like may be used. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oil and injectable organic esters such as ethyloleate. Aqueous carriers include water, alcoholic/aqueous solutions, saline solutions, parenteral vehicles such as sodium chloride, Ringer's dextrose, *etc.* Intravenous
5 vehicles include fluid and nutrient replenishers. Preservatives include antimicrobial agents, anti-oxidants, chelating agents and inert gases. The pH and exact concentration of the various components the pharmaceutical composition are adjusted according to well known parameters.

10 Additional formulations which are suitable for oral administration. Oral formulations include such typical excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate and the like. The compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders. When
15 the route is topical, the form may be a cream, ointment, salve or spray.

An effective amount of the therapeutic agent is determined based on the intended goal, for example (i) inhibition of tumor cell proliferation, (ii) elimination or killing of tumor cells, (iii) vaccination, or (iv) gene transfer for long term expression
20 of a therapeutic gene. The term "unit dose" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the therapeutic composition calculated to produce the desired responses, discussed above, in association with its administration, *i.e.*, the appropriate route and treatment regimen. The quantity to be administered, both according to number of treatments and unit
25 dose, depends on the subject to be treated, the state of the subject and the result desired. Multiple gene therapeutic regimens are expected, especially for adenovirus.

In certain embodiments of the present invention, an adenoviral vector encoding a tumor suppressor gene will be used to treat cancer patients. Typical
30 amounts of an adenovirus vector used in gene therapy of cancer is 10^3 - 10^{15} PFU/dose, (10^3 , 10^4 , 10^5 , 10^6 , 10^7 , 10^8 , 10^9 , 10^{10} , 10^{11} , 10^{12} , 10^{13} , 10^{14} , 10^{15}) wherein the dose

may be divided into several injections at different sites within a solid tumor. The treatment regimen also may involve several cycles of administration of the gene transfer vector over a period of 3-10 weeks. Administration of the vector for longer periods of time from months to years may be necessary for continual therapeutic benefit.

In another embodiment of the present invention, an adenoviral vector encoding a therapeutic gene may be used to vaccinate humans or other mammals. Typically, an amount of virus effective to produce the desired effect, in this case vaccination, would be administered to a human or mammal so that long term expression of the transgene is achieved and a strong host immune response develops. It is contemplated that a series of injections, for example, a primary injection followed by two booster injections, would be sufficient to induce an long term immune response. A typical dose would be from 10^6 to 10^{15} PFU/injection depending on the desired result. Low doses of antigen generally induce a strong cell-mediated response, whereas high doses of antigen generally induce an antibody-mediated immune response. Precise amounts of the therapeutic composition also depend on the judgment of the practitioner and are peculiar to each individual.

11. Examples

The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

EXAMPLE 1**Materials and Methods****A) Cells**

293 cells (human epithelial embryonic kidney cells) from the Master Cell
5 Bank were used for the studies.

B) Media

Dulbecco's modified Eagle's medium (DMEM, 4.5g/L glucose) + 10% fetal
bovine serum (FBS) was used for the cell growth phase. For the virus production
10 phase, the FBS concentration in DMEM was lowered to 2%.

C) Virus

AdCMVp53 is a genetically engineered, replication-incompetent human type 5
adenovirus expressing the human wild type p53 protein under control of the
15 cytomegalovirus (CMV) immediate early promoter.

D) Celligen bioreactor

A Celligen bioreactor (New Brunswick Scientific, Co. Inc.) with 5 L total
volume (3.5 L working volume) was used to produce virus supernatant using
20 microcarrier culture. 13g/L glass coated microcarrier (SoloHill) was used for
culturing cells in the bioreactor.

E) Production of virus supernatant in the Celligen bioreactor

293 cells from master cell bank (MCB) were thawed and expanded into
25 Cellfactories (Nunc). Cells were generally split at a confluence of about 85-90%.
Cells were inoculated into the bioreactor at an inoculation concentration of 1×10^5
cells/ml. Cells were allowed to attach to the microcarriers by intermittent agitation.
Continuous agitation at a speed of 30 rpm was started 6-8 hr post cell inoculation.
Cells were cultured for 7 days with process parameters set at pH=7.20, dissolved
30 oxygen (DO)=60% of air saturation, temperature=37°C. On day 8, cells were infected
with AdCMVp53 at an MOI of 5. Fifty hr post virus infection, agitation speed was

increased from 30 rpm to 150 rpm to facilitate cell lysis and release of the virus into the supernatant. The virus supernatant was harvested 74 hr post-infection. The virus supernatant was then filtered for further concentration/diafiltration.

5 ***F) Cellcube™ bioreactor system***

A Cellcube™ bioreactor system (Corning-Costar) was also used for the production of AdCMVp53 virus. It is composed of a disposable cell culture module, an oxygenator, a medium recirculation pump and a medium pump for perfusion. The cell culture module used has a culture surface area of 21,550 cm² (1 mer).

10

G) Production of virus in the Cellcube™

293 cells from master cell bank (MCB) were thawed and expanded into Cellfactories (Nunc). Cells were generally split at a confluence of about 85-90%. Cells were inoculated into the Cellcube™ according to the manufacturer's
15 recommendation. Inoculation cell densities were in the range of $1-1.5 \times 10^4/\text{cm}^2$. Cells were allowed to grow for 7 days at 37°C under culture conditions of pH=7.20, DO=60% air saturation. Medium perfusion rate was regulated according to the glucose concentration in the Cellcube™. One day before viral infection, medium for perfusion was changed from DMEM+10% FBS to DMEM+2% FBS. On day 8, cells
20 were infected with AdCMVp53 virus at a multiplicity of infection (MOI) of 5. Medium perfusion was stopped for 1 hr immediately after infection then resumed for the remaining period of the virus production phase. Culture was harvested 45-48 hr post-infection.

25 ***H) Lysis solution***

Tween-20 (Fisher Chemicals) at a concentration of 1% (v/v) in 20 mM Tris + 0.25 M NaCl + 1mM MgCl₂, pH=7.50 buffer was used to lyse cells at the end of the virus production phase in the Cellcube™.

I) Clarification and filtration

Virus supernatant from the Celligen bioreactor and virus solution from the Cellcube™ were first clarified using a depth filter (Preflow, GelmanSciences), then was filtered through a 0.8/0.22 µm filter (SuporCap 100, GelmanSciences).

5

J) Concentration/diafiltration

Tangential flow filtration (TFF) was used to concentrate and buffer exchange the virus supernatant from the Celligen bioreactor and the virus solution from the Cellcube™. A Pellicon II mini cassette (Millipore) of 300 K nominal molecular weight cut off (NMWC) was used for the concentration and diafiltration. Virus solution was first concentrated 10-fold. This was followed by 4 sample volume of buffer exchange against 20 mM Tris + 1.0 M NaCl + 1mM MgCl₂, pH=9.00 buffer using the constant volume diafiltration method.

15 Similar concentration/diafiltration was carried out for the column purified virus. A Pellicon II mini cassette of 100 K NMWC was used instead of the 300 K NMWC cassette. Diafiltration was done against 20 mM Tris + 0.25 M NaCl + 1mM MgCl₂, pH=9.00 buffer or Dulbecco's phosphate buffered saline (DPBS).

20 **K) Benzonase treatment**

The concentrated/diafiltrated virus solution was treated with Benzonase™ (American International Chemicals) at a concentration of 100 u/ml, room temperature overnight to reduce the contaminating nucleic acid concentration in the virus solution.

25 **L) CsCl gradient ultracentrifugation**

Crude virus solution was purified using double CsCl gradient ultracentrifugation using a SW40 rotor in a Beckman ultracentrifuge (XL-90). First, 7 ml of crude virus solution was overlaid on top of a step CsCl gradient made of equal volume of 2.5 ml of 1.25 g/ml and 1.40 g/ml CsCl solution, respectively. The CsCl gradient was centrifuged at 35,000 rpm for 1 hr at room temperature. The virus band
30 at the gradient interface was recovered. The recovered virus was then further purified

through a isopicnic CsCl gradient. This was done by mixing the virus solution with at least 1.5-fold volume of 1.33 g/ml CsCl solution. The CsCl solution was centrifuged at 35,000 rpm for at least 18 hr at room temperature. The lower band was recovered as the intact virus. The virus was immediately dialyzed against 20mM Tris + 1mM MgCl₂, pH=7.50 buffer to remove CsCl. The dialyzed virus was stored at -70°C for future use.

M) Ion exchange chromatography (IEC) purification

The Benzonase treated virus solution was purified using IEC. Strong anionic resin Toyopearl SuperQ 650M (Tosohaas) was used for the purification. A FPLC system (Pharmacia) with a XK16 column (Pharmacia) were used for the initial method development. Further scale-up studies were carried out using a BioPilot system (Pharmacia) with a XK 50 column (Pharmacia). Briefly, the resin was packed into the columns and sanitized with 1 N NaOH, then charged with buffer B which was followed by conditioning with buffer A. Buffers A and B were composed of 20 mM Tris + 0.25 M NaCl + 1mM MgCl₂, pH=9.00 and 20 mM Tris + 2M NaCl + 1 mM MgCl₂, pH=9.00, respectively. Viral solution sample was loaded onto the conditioned column, followed by washing the column with buffer A until the UV absorption reached base line. The purified virus was eluted from the column by using a 10 column volume of linear NaCl gradient.

N) HPLC analysis

A HPLC analysis procedure was developed for evaluating the efficiency of virus production and purification. Tris(hydroxymethyl)aminomethane (tris) was obtained from FisherBiotech (Cat# BP154-1; Fair Lawn, New Jersey, U. S. A.); sodium chloride (NaCl) was obtained from Sigma (Cat# S-7653, St. Louis, MO, U. S.A.). Both were used directly without further purification. HPLC analyses were performed on an Analytical Gradient System from Beckman, with Gold Workstation Software (126 binary pump and 168 diode array detector) equipped with an anion-exchange column from TosoHaas (7.5 cm × 7.5 mm ID, 10 µm particle size, Cat# 18257). A 1-ml Resource Q (Pharmacia) anion-exchange column was used to

evaluate the method developed by Huyghe *et al.* using their HEPES buffer system. This method was only tried for the Bioreactor system.

5 The buffers used in the present HPLC system were *Buffer A*: 10 mM tris buffer, pH 9.0. *Buffer B*: 1.5 M NaCl in buffer A, pH 9.0. The buffers were filtered through a 0.22 μ m bottle top filter by Corning (Cat# 25970-33). All of the samples were filtered through a 0.8/0.22 μ m Acrodisc PF from Gelman Sciences (Cat# 4187) before injection.

10 The sample is injected onto the HPLC column in a 60-100 μ l volume. After injection, the column (TosoHaas) is washed with 20% B for 3 min at a flow rate of 0.75 ml/min. A gradient is then started, in which B is increased from 20% to 50% over 6 min. Then the gradient is changed from 50% to 100% B over 3 min, followed by 100% B for 6 min. The salt concentration is then changed back stepwise to 20%
15 again over 4 min, and maintained at 20% B for another 6 min. The retention time of the Adp53 is 9.5 ± 0.3 min with $A_{260}/A_{280} \cong 1.26 \pm 0.03$. Cleaning of the column after each chromatographic run is accomplished by injecting 100 μ l of 0.15 M NaOH and then running the gradient.

20

EXAMPLE 2

Effect of medium perfusion rate in Cellcube™ on virus production and purification

For a perfusion cell culture system, such as the Cellcube™, medium perfusion
25 rate plays an important role on the yield and quality of product. Two different medium perfusion strategies were examined. One strategy was to keep the glucose concentration in the Cellcube™ ≥ 2 g/L (high perfusion rate). The other one was to keep the glucose concentration ≥ 1 g/L (low medium perfusion rate).

30 No significant changes in the culture parameters, such as pH, DO, was observed between the two different perfusion rates. Approximately equivalent amount of crude viruses (before purification) were produced after harvesting using 1%

Tween-20 lysis solution as shown in Table 5. However, dramatic difference was seen on the HPLC profiles of the viral solutions from the high and low medium perfusion rate production runs.

5 **TABLE 5. Effect of medium glucose concentration on virus yield**

Glucose concentration (g/L)	≥ 2.0	≥ 1.0
Crude virus yield (PFU)	4×10^{12}	4.9×10^{12}

As shown in FIG. 1, a very well separated virus peak (retention time 9.39 min)
10 was produced from viral solution using low medium perfusion rate. It was found that virus with adequate purity and biological activity was attained after a single step ion exchange chromatographic purification of the virus solution produced under low medium perfusion rate. On the other hand, no separated virus peak in the retention time of 9.39 min was observed from viral solution produced using high medium
15 perfusion rate. This suggests that contaminants which have the same elution profile as the virus were produced under high medium perfusion rate. Although the nature of the contaminants is not yet clear, it is expected that the contaminants are related to the increased extracellular matrix protein production under high medium perfusion rate (high serum feeding) from the producer cells. This poor separation characteristic seen
20 on the HPLC created difficulties for process IEC purification as shown in the following Examples. As a result, medium perfusion rate used during the cell growth and the virus production phases in the Cellcube™ has a significant effect on the downstream IEC purification of the virus. Low medium perfusion rate is recommended. This not only produces easy to purify crude product but also offers
25 more cost-effective production due to the reduced medium consumption.

EXAMPLE 3

Methods of cell harvest and lysis

Based on previous experience, the inventors first evaluated the freeze-thaw
5 method. Cells were harvested from the Cellcube™ 45-48 hr post-infection. First, the
Cellcube™ was isolated from the culture system and the spent medium was drained.
Then, 50 mM EDTA solution was pumped into the Cube to detach the cells from the
culture surface. The cell suspension thus obtained was centrifuged at 1,500 rpm
(Beckman GS-6KR) for 10 min. The resultant cell pellet was resuspended in
10 Dulbecco's phosphate buffered saline (DPBS). The cell suspension was subjected to
5 cycles of freeze/thaw between 37°C water bath and dry-ice ethanol bath to release
virus from the cells. The crude cell lysate (CCL) thus generated was analyzed on
HPLC.

15 FIG. 2 shows the HPLC profile. No virus peak is observed at retention time of
9.32 min. Instead, two peaks at retention times of 9.11 and 9.78 min are produced.
This profile suggests that the other contaminants having similar elution time as the
virus exist in the CCL and interfere with the purification of the virus. As a result,
very low purification efficiency was observed when the CCL was purified by IEC
20 using FPLC.

In addition to the low purification efficiency, there was a significant product
loss during the cell harvest step into the EDTA solution as indicated in Table 6.
Approximately 20% of the product was lost into the EDTA solution which was
25 discarded. In addition, about 24% of the crude virus product is present in the spent
medium which was also discarded. Thus, only 56% of the crude virus product is in
the CCL. Furthermore, freeze-thaw is a process of great variation and very limited
scaleability. A more efficient cell lysis process with less product loss needed to be
developed.

TABLE 6. Loss of virus during EDTA harvest of cells from Cellcube™

	Waste		Crude product	Total crude product (PFU)
	Spent Medium	EDTA harvest Solution	Crude cell lysate	
Volume (ml)	2800	2000	82	-
Titer (PFU/ml)	2.6×10^8	3×10^8	2×10^{10}	-
Total virus (PFU)	7.2×10^{11}	6×10^{11}	1.64×10^{12}	3×10^{12}
Percentage	24%	20%	56%	

5

Data was generated from 1 mer Cellcube™.

TABLE 7. Evaluation of non-ionic detergents for cell lysis

Detergents	Concentrations (w/v)	Chemistry	Comments
Thesit	1%	Dodecylpoly(ethylene glycol ether) _n n=9-10	Large Precipitate
	0.5%		
	0.1%		
NP-40	1%	Ethylphenolpoly (ethylene-glycolether) _n n=9-11	Large precipitate
	0.5%		
	0.1%		
Tween-20	1%	Poly(oxyethylene) _n -sorbitan-monolaurate n=20	Small precipitate
	0.5%		
	0.1%		
Brij-58	1%	Cetyl poly (ethyleneglycolether) _n n=20	Cloudy Solution
	0.5%		
	0.1%		
Triton X-100	1%	Octylphenolpoly(ethyleneglycolether) _n n=10	Large precipitate
	0.5%		
	0.1%		

Detergents have been used to lyse cells to release intracellular organelles. Consequently, the inventors evaluated the detergent lysis method for the release of adenovirus. Table 7 lists the 5 different non-ionic detergents that were evaluated for cell lysis. Cells were harvested from the Cellcube™ 48 hr post-infection using 50 mM EDTA. The cell pellet was resuspended in the different detergents at various concentrations listed in Table 7.

Cell lysis was carried out at either room temperature or on ice for 30 min. Clear lysis solution was obtained after centrifugation to remove the precipitate and cellular debris. The lysis solutions were treated with Benzonase and then analyzed by HPLC. FIG. 3 shows the HPLC profiles of lysis solutions from the different detergents. Thesit and NP-40 performed similarly as Triton X-100. Lysis solution generated from 1% Tween-20 gave the best virus resolution with the least virus resolution being observed with Brij-58. More efficient cell lysis was found at detergent concentration of 1% (w/v). Lysis temperature did not contribute significantly to the virus resolution under the detergent concentrations examined. For the purpose of process simplicity, lysis at room temperature is recommended. Lysis solution composed of 1% Tween-20 in 20 mM Tris + 0.25M NaCl + 1 mM MgCl₂, pH=7.50 was employed for cell lysis and virus harvest in the Cellcube™.

EXAMPLE 4

Effects of concentration/diafiltration on virus recovery

Virus solution from the lysis step was clarified and filtered before concentration/ diafiltration. TFF membranes of different NMWCs, including 100K, 300K, 500K, and 1000K, were evaluated for efficient concentration/diafiltration. The highest medium flux with minimal virus loss to the filtrate was obtained with a membrane of 300K NMWC. Bigger NMWC membranes offered higher medium flux, but resulted in greater virus loss to the filtrate, while smaller NMWC membranes achieved an insufficient medium flux. Virus solution was first concentrated 10-fold, which was followed by 4 sample volumes of diafiltration against 20 mM Tris + 0.25

M NaCl + 1 mM MgCl₂, pH=9.00 buffer using the constant volume method. During the concentration/diafiltration process, pressure drop across the membrane was kept ≤ 5 psi. Consistent, high level virus recovery was demonstrated during the concentration/diafiltration step as indicated in Table 8.

TABLE 8. Concentration/diafiltration of crude virus solution

	Titer (PFU/ml)		Volume (ml)		Total virus (PFU)		Recovery	
	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2
Before conc./diafl.	2.6×10^9	2×10^9	1900	2000	4.9×10^{12}	4×10^{12}		
Post conc./diafl.	2.5×10^{10}	1.7×10^{10}	200	200	5×10^{12}	3.4×10^{12}	102%	85%
Conc. Factor			9.5	10				
Filtrate	5×10^5	1×10^6	3000	3000	1.5×10^9	3×10^9		

EXAMPLE 5**Effect of salt addition on Benzonase treatment**

Virus solution after concentration/diafiltration was treated with Benzonase (nuclease) to reduce the concentration of contaminating nucleic acid in virus solution. Different working concentrations of Benzonase, which included 50, 100, 200, 300 units/ml, were evaluated for the reduction of nucleic acid concentrations. For the purpose of process simplicity, treatment was carried out at room temperature overnight. Significant reduction in contaminating nucleic acid that is hybridizable to human genomic DNA probe was seen after Benzonase treatment.

Table 9 shows the reduction of nucleic acid concentration before and after Benzonase treatment. Virus solution was analyzed on HPLC before and after Benzonase treatment. As shown in FIG. 4A and FIG. 4B, dramatic reduction in the contaminating nucleic acid peak was observed after Benzonase treatment. This is in agreement with the result of the nucleic acid hybridization assay. Because of the effectiveness, a Benzonase concentration of 100 u/ml was employed for the treatment of the crude virus solution.

Table 9. Reduction of contaminating nucleic acid concentration in virus solution

	Before Treatment	After Treatment	Reduction
Contaminating nucleic acid concentration	200 µg/ml	10 ng/ml	2×10^4 -fold

Treatment condition: Benzonase concentration: 100 u/ml, temperature: room temperature, time: overnight.

Considerable change in the HPLC profile was observed pre- and post-Benzonase treatment. No separated virus peak was detected at retention time of 9.33 min after Benzonase treatment. At the same time, a major peak with high 260 nm adsorption at retention time of 9.54 min was developed. Titer assay results indicated that Benzonase treatment did not negatively affect the virus titer and virus remained intact and infectious after Benzonase treatment. It was reasoned that cellular nucleic acid released during the cell lysis step interacted with virus and either formed aggregates with the virus or adsorbed onto the virus surface during Benzonase treatment.

To minimize the possible nucleic acid virus interaction during Benzonase treatment, different concentrations of NaCl was added into the virus solution before Benzonase treatment. No dramatic change in the HPLC profile occurred after Benzonase treatment in the presence of 1 M NaCl in the virus solution. FIG. 5 shows the HPLC profile of virus solution after Benzonase treatment in the presence of 1M NaCl. Unlike that shown in FIG. 4B, virus peak at retention time of 9.35 min still exists post Benzonase treatment. This result indicates that the presence of 1M NaCl prevents the interaction of nucleic acid with virus during Benzonase treatment and facilitates the further purification of virus from contaminating nucleic acid.

EXAMPLE 6

Ion exchange chromatographic purification

The presence of negative charge on the surface of adenovirus at physiological pH conditions prompted evaluation of anionic ion exchangers for adenovirus purification. The strong anionic ion exchanger Toyopearl Super Q 650M was used for the development of a purification method. The effects of NaCl concentration and pH of the loading buffer (buffer A) on virus purification was evaluated using the FPLC system.

A) *Method development*

For ion exchange chromatography, buffer pH is one of the most important parameters and can have dramatic influence on the purification efficiency. In reference to the medium pH and conductivity used during virus production, the inventors formulated 20 mM Tris+1mM MgCl₂+ 0.2M NaCl, pH=7.50 as buffer A. A
5 XK16 column packed with Toyopearl SuperQ 650M with a height of 5 cm was conditioned with buffer A.

A sample of 5 ml of Benzonase treated concentrated/diafiltrated virus
10 supernatant from the Celligen bioreactor was loaded onto the column. After washing the column, elution was carried out with a linear gradient of over 10 column volumes of buffer B formulation to reach mM Tris + 1mM MgCl₂ + 2M NaCl, pH=7.50.

FIG. 6 shows the elution profile. Three peaks were observed during elution
15 without satisfactory separation among them. Control study performed with 293 cell conditioned medium (with no virus) showed that the first two peaks are virus related. To further improve the separation efficiency, the effect of buffer pH was evaluated. Buffer pH was increased to 9.00 while keeping other conditions constant. Much improved separation, as shown in FIG. 7, was observed as compared to that of buffer
20 pH of 7.50. Fractions #3, #4, and #8 were analyzed on HPLC.

As shown in FIG. 8, the majority of virus was found in fraction #4, with no virus being detected in fractions #3 and #8. Fraction #8 was found to be mainly composed of contaminating nucleic acid. However, the purification was still not
25 optimal. There is overlap between fractions #3 and #4 with contaminants still detected in fraction #4.

Based on the chromatogram in FIG. 7, it was inferred that further improvement of virus purification could be achieved by increasing the salt
30 concentration in buffer A. As a result, the contaminants present in the fraction #3, which is prior to the virus peak, can be shifted to the flow through fraction. The NaCl

concentration in buffer A was increased to 0.3 M while keeping other conditions constant. FIG. 9 shows the elution profile under the condition of 0.3 M NaCl in buffer A.

5 Dramatic improvement in purification efficiency was achieved. As expected the contaminant peak observed in FIG. 7 was eliminated under the increased salt condition. Samples from crude virus sup, flow through, peak #1, and peak #2 were analyzed on HPLC and the results are shown in FIG. 10. No virus was detected in the flow through fraction. The majority of the contaminants present in the crude material
10 were found in the flow through. HPLC analysis of peak #1 showed a single well defined virus peak. This HPLC profile is equivalent to that obtained from double CsCl gradient purified virus. Peaks observed at retention times of 3.14 and 3.61 min in CsCl gradient purified virus are glycerol related peaks. The purified virus has a A260/A280 ratio of 1.27 ± 0.03 . This similar to the value of double CsCl gradient
15 purified virus as well as the results reported by Huyghe *et al.* (1996). Peak #2 is composed mainly of contaminating nucleic acid. Based on the purification result, the inventors proposed the following method for IEC purification of adenovirus sup from the bioreactor.

20 Buffer A: 20 mM Tris + 1mM $MgCl_2$ + 0.3M NaCl, pH=9.00
 Buffer B: 20 mM Tris + 1mM $MgCl_2$ + 2M NaCl, pH=9.00
 Elution: 10 column volume linear gradient

B) Method scale-up

25 Following the development of the method, purification was scaled-up from the XK16 column (1.6 cm I.D.) to a XK50 column (5cm I.D., 10-fold scale-up) using the same purification method. A similar elution profile was achieved on the XK50 column as shown in FIG. 11. The virus fraction was analyzed on HPLC, which indicated equivalent virus purity to that obtained from the XK16 column.

During the scale-up studies, it was found that it was more convenient and consistent to use conductivity to quantify the salt concentration in buffer A. The optimal conductivity of buffer A is in the range of 25 ± 2 mS/cm at approximately room temperature (21°C). Samples produced during the purification process together
5 with double CsCl purified virus were analyzed on SDS-PAGE.

As shown in FIG. 12, all the major adenovirus structure proteins are detected on the SDS-PAGE. The IEC purified virus shows equivalent staining as that of the double CsCl purified virus. Significant reduction in bovine serum albumin (BSA)
10 concentration was achieved during purification. The BSA concentration in the purified virus was below the detection level of the western blot assay as shown in FIG. 13.

The reduction of contaminating nucleic acid concentration in virus solution
15 during the purification process was determined using nucleic acid slot blot. ^{32}P labeled human genomic DNA was used as the hybridization probe (because 293 cells are human embryonic kidney cells). Table 10 shows the nucleic acid concentration at different stages of the purification process. Nucleic acid concentration in the final purified virus solution was reduced to 60 pg/ml, an approximate 3.6×10^6 -fold
20 reduction compared to the initial virus supernatant. Virus titer and infectious to total particle ratio were determined for the purified virus and the results were compared to that from double CsCl purification in Table 9. Both virus recovery and particle/PFU ratio are very similar between the two purification methods. The titer of the column purified virus solution can be further increased by performing a concentration step.

TABLE 10. Removal of contaminating nucleic acids during purification

Steps during purification	Contaminating nucleic acid concentration
Virus supernatant from bioreactor	220 µg/ml
Concentrated/diafiltrated sup	190 µg/ml
Sup post Benzonase treatment (O/N, RT, 100 u/ml)	10 ng/ml
Purified virus from column	210 pg/ml
Purified virus post concentration/diafiltration	60 pg/ml
CsCl purified virus	800 pg/ml

5

EXAMPLE 7**Other purification methods**

In addition to the strong anionic ion exchange chromatography, other modes of chromatographic methods, were also evaluated for the purification of AdCMVp53 virus (e.g. size exclusion chromatography, hydrophobic interaction chromatography, cation exchange chromatography, or metal ion affinity chromatography). Compared to the Toyopearl Super Q, all those modes of purification offered much less efficient purification with low product recovery. Therefore, Toyopearl Super Q resin is recommended for the purification of AdCMVp53. However, other quaternary ammonium chemistry based strong anionic exchangers are likely to be suitable for the purification of AdCMVp53 with some process modifications.

10

15

EXAMPLE 8

Purification of crude AdCMVp53 virus generated from Cellcube™

Two different production methods were developed to produce AdCMVp53 virus. One was based on microcarrier culture in a stirred tank bioreactor. The other was based on a Cellcube™ bioreactor. As described above, the purification method was developed using crude virus supernatant generated from the stirred tank bioreactor. It was realized that although the same medium, cells and viruses were used for virus production in both the bioreactor and the Cellcube™, the culture surface onto which cells attached was different.

In the bioreactor, cells were grown on a glass coated microcarrier, while in the Cellcube™ cells were grown on proprietary treated polystyrene culture surface. Constant medium perfusion was used in the Cellcube™, on the other hand, no medium perfusion was used in the bioreactor. In the Cellcube™, the crude virus product was harvested in the form of virally infected cells, which is different from the virus supernatant harvested from the bioreactor.

Crude cell lysate (CCL), produced after 5 cycles freeze-thaw of the harvested virally infected cells, was purified by IEC using the above described method. Unlike the virus supernatant from the bioreactor, no satisfactory purification was achieved for the CCL material generated from the Cellcube™. FIG. 14 shows the chromatogram. The result suggests that crude virus solution generated from the Cellcube™ by freeze-thawing harvested cells is not readily purified by the IEC method.

Other purification methods, including hydrophobic interaction and metal chelate chromatography, were examined for the purification of virus in CCL. Unfortunately, no improvement in purification was observed by either method. Considering the difficulties of purification of virus in CCL and the disadvantages associated with a freeze-thaw step in the production process, the inventors decided to explore other cell lysis methods.

A) Purification of crude virus solution in lysis buffer

As described in Examples 1 and 3, HPLC analysis was used to screen different detergent lysis methods. Based on the HPLC results, 1% Tween-20 in 20 mM Tris +0.25 M NaCl +1 mM MgCl₂, pH=7.50 buffer was employed as the lysis buffer. At the end of the virus production phase, instead of harvesting the infected cells, the lysis buffer was pumped into the Cellcube™ after draining the spent medium. Cells were lysed and virus released into the lysis buffer by incubating for 30 min.

After clarification and filtration, the virus solution was concentrated/diafiltrated and treated with Benzonase to reduce the contaminating nucleic acid concentration. The treated virus solution was purified by the method developed above using Toyopearl SuperQ resin. Satisfactory separation, similar to that obtained using virus supernatant from the bioreactor, was achieved during elution. FIG. 15 shows the elution profile. However, when the virus fraction was analyzed on HPLC, another peak in addition to the virus peak was detected. The result is shown in FIG. 16A.

To further purify the virus, the collected virus fraction was re-purified using the same method. As shown in FIG. 16B, purity of the virus fraction improved considerably after the second purification. Metal chelate chromatography was also evaluated as a candidate for the second purification. Similar improvement in virus purity as seen with the second IEC was achieved. However, because of its simplicity, IEC is preferred as the method of choice for the second purification.

As described above in Example 2, medium perfusion rate employed during the cell growth and virus production phases has a considerable impact on the HPLC separation profile of the Tween-20 crude virus harvest. For crude virus solution produced under high medium perfusion rate, two ion exchange columns are required to achieve the required virus purity.

Based on the much improved separation observed on HPLC for virus solution produced under low medium perfusion rate, it is likely that purification through one ion exchange column may achieve the required virus purity. FIG. 17 shows the elution profile using crude virus solution produced under low medium perfusion rate.

- 5 A sharp virus peak was attained during elution. HPLC analysis of the virus fraction indicates virus purity equivalent to that of CsCl gradient purified virus after one ion exchange chromatography step. FIG. 18 shows the HPLC analysis result.

The purified virus was further analyzed by SDS-PAGE, western blot for BSA,
10 and nucleic acid slot blot to determine the contaminating nucleic acid concentration. The analysis results are given in FIG. 19A, FIG. 19B and FIG. 19C, respectively. All those analyses indicate that the column purified virus has equivalent purity compared to the double CsCl gradient purified virus. Table 11 shows the virus titer and recovery before and after the column purification. For comparison purposes, the
15 typical virus recovery achieved by double CsCl gradient purification was also included. Similar virus recoveries were achieved by both methods.

TABLE 11. Comparison of IEC and double CsCl gradient ultracentrifugation purification of AdCMVp53 from Cellcube™

20

	Titer (PFU/ml)	A260/A280	Particle/PFU	Recovery
IEC	1×10^{10}	1.27	36	63%
Ultracentrifugation	2×10^{10}	1.26	38	60%

A) Resin capacity study

The dynamic capacity of the Toyopearl Super Q resin was evaluated for the purification of the Tween-20 harvested virus solution produced under low medium
25 perfusion rate. One hundred ml of resin was packed in a XK50 column. Different amount of crude virus solution was purified through the column using the methods described herein.

Virus breakthrough and purification efficiency were analyzed on HPLC. FIG. 20 shows the HPLC analysis results. At a column loading factor greater than sample/column volume ratio of 2:1, purity of the virus fraction was reduced. Contaminants co-eluted with the virus. At a loading factor of greater than 3:1, breakthrough of the virus into the flow through was observed. Therefore, it was proposed that the working loading capacity of the resin be in the range of sample/column volume ratio of 1:1.

B) Concentration/diafiltration post purification

A concentration/diafiltration step after column purification serves not only to increase the virus titer, if necessary, but also to exchange to the buffer system specified for the virus product. A 300K NMWC TFF membrane was employed for the concentration step. Because of the absence of proteinacious and nucleic acid contaminants in the purified virus, very high buffer flux was achieved without noticeable pressure drop across the membrane.

Approximately 100% virus recovery was achieved during this step by changing the buffer into 20mM Tris + 1mM MgCl₂ + 0.15 M NaCl, pH=7.50. The purified virus was also successfully buffer exchanged into DPBS during the concentration/diafiltration step. The concentration factor can be determined by the virus titer that is desired in the final product and the titer of virus solution eluted from the column. This flexibility will help to maintain the consistency of the final purified virus product.

C) Evaluation of defective adenovirus in the IEC purified AdCMVp53

Due to the less than 100% packaging efficiency of adenovirus in producer cells, some defective adenoviruses generally exist in crude virus solution. Defective viruses do not have DNA packaged inside the viral capsid and therefore can be separated from intact virus on CsCl gradient ultracentrifugation based the density difference. It is likely that it would be difficult to separate the defective from the intact viruses based on ion exchange chromatography assuming both viruses have

similar surface chemistry. The presence of excessive amount of defective viruses will impact the quality of the purified product.

To evaluate the percentage of defective virus particles present, the purified and concentrated viruses were subjected to isopicnic CsCl ultracentrifugation. As shown in FIG. 21, a faint band on top of the intact virus band was observed after centrifugation. Both bands were recovered and dialyzed against 20mM Tris + 1mM MgCl₂, pH=7.50 buffer to remove CsCl. The dialyzed viruses were analyzed on HPLC and the results are shown in FIG. 22. Both viruses show similar retention time. However, the defective virus has a smaller A260/A280 ratio than that of the intact virus. This is indicative of less viral DNA in the defective virus.

The peaks seen at retention times between 3.02 to 3.48 min are produced by glycerol which is added to the viruses (10% v/v) before freezing at -70°C. The percentage of the defective virus was less than 1% of the total virus. This low percentage of defective virus is unlikely to impact the total particle to infectious virus (PFU) ratio in the purified virus product. Both viruses were analyzed by SDS-PAGE (shown in FIG. 19A). Compared to the intact viruses, defective viruses lack the DNA associated core proteins banded at 24 and 48.5 KD. This result is in agreement with the absence of DNA in defective virus.

D) Process overview of the production and purification of AdCMVp53 virus

Based on the above process development results, the inventors propose a production and purification flow chart for AdCMVp53 as shown in FIG. 23. The step and accumulative virus recovery is included with the corresponding virus yield based on a 1 mer Cellcube™. The final virus recovery is about 70±10%. This is about 3-fold higher than the virus recovery reported by Huyghe *et al.* (1996) using a DEAE ion exchanger and a metal chelate chromatographic purification procedure for the purification of p53 protein encoding adenovirus. Approximately 3×10^{12} PFU of final purified virus product was produced from a 1 mer Cellcube™. This represents a

similar final product yield compared to the current production method using double CsCl gradient ultracentrifugation for purification.

E) Scale-up

5 Successful scale-up studies have been performed with the 4 mer Cellcube™ system, and are currently underway to evaluate virus production in the 16 mer Cellcube™ system. The crude virus solution produced will be filtered, concentrated and diafiltrated using a bigger Pellicon cassette. The quality and recovery of the virus will be determined. After Benzonase treatment, the crude virus
10 solution will be purified using a 20 cm and a 30 cm BioProcess column for the 4 mer and 16 mer, respectively.

EXAMPLE 9

Improved Ad-p53 Production in Serum-Free Suspension Culture

15

Adaptation of 293 cells

293 cells were adapted to a commercially available IS293 serum-free media (Irvine Scientific; Santa Ana, CA) by sequentially lowering down the FBS concentration in T-flasks. The frozen cells in one vial of PDWB were thawed and
20 placed in 10% FBS DMEM media in T-75 flask and the cells were adapted to serum-free IS 293 media in T-flasks by lowering down the FBS concentration in the media sequentially. After 6 passages in T-75 flasks the FBS% was estimated to be about 0.019%. The cells were subcultured two more times in the T flasks before they were transferred to spinner flasks.

25

Serum-free adapted 293 cells in T flasks were adapted to suspension culture

The above serum-free adapted cells in T-flasks were transferred to a serum-free 250 mL spinner suspension culture (100 mL working volume) for the suspension culture. The initial cell density was 1.18E+5 vc/mL. During the cell culture the
30 viability decreased and the big clumps of cells were observed. After 2 more passages in T-flasks the adaptation to suspension culture was tried again. In a second attempt

the media was supplemented with heparin, at a concentration of 100mg/L, to prevent aggregation of cells and the initial cell density was increased to 5.22×10^5 vc/mL. During the cell culture there was some increase of cell density and cell viability was maintained. Afterwards the cells were subcultured in the spinner flasks for 7 more passages and during the passages the doubling time of the cells was progressively reduced and at the end of seven passages it was about 1.3 day which is comparable to 1.2 day of the cells in 10% FBS media in the attached cell culture. In the serum-free IS 293 media supplemented with heparin almost all the cells existed as individual cells not forming aggregates of cells in the suspension culture (Table 12).

TABLE 12. Serum-Free Suspension Culture: Adaptation to Suspension

Passage No.	Flask No.	Average Doubling Time (days)
11		Viability decreased
13		3.4
14		3.2
15	1	Viability decreased
heparin added	2	4.7
	3	5.0
	4	3.1
16	1	5.5
	2	4.8
	3	4.3
	4	4.3
17	1	2.9
	2	3.5
	3	2.4
	4	1.7
18	1	3.5
	2	13.1
	3	6.1
	4	3.8
19	1	2.5
	2	2.6
	3	2.3
	4	2.5
20	1	1.3 (97% viability)
	2	1.5 (99% viability)
	3	1.8 (92% viability)
	4	1.3 (96% viability)

Viral production and growth of cells in serum-free suspension culture in spinner flask

To test the production of Ad5-CMVp53 vectors in the serum-free suspension culture the above cells adapted to the serum-free suspension culture were grown in 100 mL serum-free IS293 media supplemented with 0.1% Pluronic F-68 and Heparin (100 mg/L) in 250mL spinner flasks. the cells were infected at 5 MOI when the cells reached 1.36×10^6 viable cells/mL on day 3. The supernatant was analyzed everyday for HPLC viral particles/mL after the infection. No viruses were detected other than day 3 sample. On day 3 it was 2.2×10^9 vps/mL. The pfu/mL on day 6 was $2.6 \pm 0.6 \times 10^7$ pfu/mL. The per cell pfu production was estimated to be 19 which is approximately 46 times below the attached culture in the serum-supplemented media. As a control the growth of cells was checked in the absence of an infection.

TABLE 13. Serum-Free Suspension Culture:**Viral Production and Cell Growth**

	Control viral infection	w/o Viral infection w/o media exchange	Viral infection w/ media exchange
Initial Density (vc/mL)	2.1×10^5	2.1×10^5	2.1×10^5
Cell Density at infection (vc/mL)	9.1×10^5	1.4×10^6	1.5×10^6
Volumetric viral production (pfu/mL) 6 days P.I.	NA	2.6×10^7	2.8×10^8
Volumetric viral production (HPLC vps/mL) 6 days P.I.	NA	NA	1.3×10^{10}
Per cell viral production (HPLC vps/cell)	NA	NA	1.3×10^4

Preparation of serum-free suspension adapted 293 cell banks

As described above, after it was demonstrated the cells produce the Ad-p53 vectors, the cells were propagated in the serum-free IS293 media with 0.1 % F-68 and 100 mg/L heparin in the spinner flasks to make serum-free suspension adapted cell

banks which contain $1.0\text{E}+07$ viable cells/mL/vial. To collect the cells they were centrifuged down when they were at mid-log phase growth and the viability was over 90% and resuspended in the serum-free, supplemented IS293 media and centrifuged down again to wash out the cells. Then the cells were resuspended again in the cryopreservation media which is cold IS293 with 0.1% F-68, 100 mg/L heparin, 10% DMSO and 0.1% methylcellulose resulting in $1\text{E}+07$ viable cells/mL. The cell suspension was transferred to sterile cryopreservation vials and they were sealed and frozen in cryocontainer at -70°C overnight. The vials were transferred to liquid nitrogen storage. The mycoplasma test was negative.

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To revive the frozen cells one vial was thawed into the 50 mL serum-free IS293 media with 0.1% F-68 and 100 mg/L heparin in a T-150. Since then the cultures were subcultured three times in 250 mL spinner flasks. In the other study one vial was thawed into 100 mL serum-free, supplemented IS293 media in a 250 mL spinner flask. Since then these were subcultured in serum-free spinner flasks 2 times. In both of the studies the cells grew very well.

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Media replacement and viral production in serum-free suspension culture in spinner flask

In the previous serum-free viral production in the suspension culture in the spinner flask the per cell viral production was too low for the serum-free suspension production to be practical. It was supposed that this might be due to the depletion of nutrients and/or the production of inhibitory byproducts. To replace the spent media with fresh serum-free, supplemented IS293 media the cells were centrifuged down on day 3 and resuspended in a fresh serum-free IS-293 medium supplemented with F-68 and heparin (100 mg/L) and the resulting cell density was $1.20\text{E}+06$ vc/mL and the cells were infected with Ad5-CMVp53 vectors at 5 MOI. The extracellular HPLC vps/mL was $7.7\text{E}+09$ vps/mL on day 3, $1.18\text{E}+10$ vps/mL on day 4, $1.2\text{E}+10$ vps/mL on day 5 and $1.3\text{E}+10$ vps/mL on day 6 and the pfu/mL on day 6 was $2.75\pm 0.86\text{E}+08$ tvps/mL. The ratio of HPLC viral particles to pfus was about 47. Also the cells have been centrifuged down and lysed with the same type of the detergent lysis

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buffer as used in the harvest of CellCube. The cellular HPLC vps/mL was $1.6\text{E}+10$ vps/mL on day 2, $6.8\text{E}+09$ vps/mL on day 3, $2.2\text{E}+09$ vps/mL on day 4, $2.24\text{E}+09$ vps/mL on day 5 and $2.24\text{E}+09$ vps/mL on day 6.

5 The replacement of the spent media with a fresh serum-free, supplemented IS 293 media resulted in the significant increase in the production of Ad-p53 vectors. The media replacement increased the production of extracellular HPLC viral particles 3.6 times higher above the previous level on day 3 and the production of extracellular pfu titer ten times higher above the previous level on day 6. Per cell production of
10 Ad-p53 vectors was estimated to be approximately $1.33\text{E}+04$ HPLC vps.

 The intracellular HPLC viral particles peaked on day 2 following the infection and then the particle numbers decreased. In return the extracellular viral particles increased progressively to the day 6 of harvest. Almost all the Ad-p53 vectors were
15 produced for the 2 days following the infection and intracellularly localized and then the viruses were released outside of the cells. Almost half of the viruses were released outside of the cells into the supernatant between day 2 and day 3 following the infection and the rate of release decreased as time goes on.

20 All the cells infected with Ad-p53 vectors lost their viability at the end of 6 days after the infection while the cells in the absence of infection was 97% viable. In the presence of infection the pH of the spent media without the media exchange and with the media exchange was 6.04 and 5.97, respectively, while the one in the absence of the infection was 7.00 (Table 12).

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Viral production and cell culture in stirred bioreactor with media replacement and gas overlay

 To increase the production of Ad-p53 vectors, a 5L CelliGen bioreactor was used to provide a more controlled environment. In the 5 L CelliGen bioreactor the pH
30 and the dissolved oxygen as well as the temperature was controlled. Oxygen and carbon dioxide gas was connected to the solenoid valve for oxygen supply and the pH

adjustment, respectively. For a better mixing while generating low shear environment a marine-blade impeller was implemented. Air was supplied all the time during the operation to keep a positive pressure inside the bioreactor.

5 To inoculate the bioreactor a vial of cells was thawed into 100 mL serum-free media in a 250 mL spinner flask and the cells were expanded in 250 or 500 mL spinner flasks. 800 mL cell inoculum, grown in 500 mL flasks, was mixed with 2700 mL fresh media in a 10 L carboy and transferred to the CelliGen bioreactor by gas pressure. The initial working volume of the CelliGen bioreactor was about 3.5 L
10 culture. The agitation speed of the marine-blade impeller was set at 80 rpm, the temperature at 37°C, pH at 7.1 at the beginning and 7.0 after the infection and the DO at 40% all the time during the run.

The initial cell density was 4.3E+5 vc/mL (97% viability) and 4 days later
15 when the cell density reached to 2.7E+6 vc/mL (93% viability) the cells were centrifuged down and the cells were resuspended in a fresh media and transferred to the CelliGen bioreactor. After the media exchange the cell density was 2.1E+6 vc/mL and the cells were infected at MOI of 10. Since then the DO dropped to below 40%. To keep the DO above 40%, about 500 mL of culture was withdrawn from the
20 CelliGen bioreactor to lower down the oxygen demand by the cell culture and the upper marine-blade was positioned close to the interface between the gas and the liquid phase to improve the oxygen transfer by increasing the surface renewal. Since then the DO could be maintained above 40% until the end of the run.

25 For pH control, CO₂ gas was used to acidify the cell culture and 1 N NaHCO₃ solution to make the cell culture alkaline. The pH control was initially set at 7.10. The initial pH of the cell culture was about pH 7.41. Approximately 280 mL 1N NaHCO₃ solution was consumed until the pH of cell culture stabilized around pH 7.1. After the viral infection of the cell culture, the pH control was lowered down to pH
30 7.0 and the CO₂ gas supply line was closed off to reduce the consumption of NaHCO₃ solution. The consumption of too much NaHCO₃ solution for pH adjustment would

increase the cell culture volume undesirably. Since then 70 mL 1N NaHCO₃ solution was consumed and the pH was in the range between 7.0 and 7.1 most of the time during the run. The temperature was controlled between 35°C and 37°C.

5 After the infection the viability of the cells decreased steadily until day 6 of harvest after the infection. On the harvest day none of the cells was viable. The volumetric viral production of the CelliGen bioreactor was 5.1E+10 HPLC vps/mL compared to the 1.3E+10 vps/mL in the spinner flask. The controlled environment in the CelliGen bioreactor increased the production of Ad-p53 vectors 4-fold compared to the spinner flasks with media replacement. This is both due to the increase of the
10 cell density at the time of infection from 1.2E+6 to 2.1E+6 vc/mL and the increase of per cell viral production from 1.3E+4 to 2.5E +4 vps/mL. The 2.5E+4 vps/mL is comparable to the 3.5E+4 vps/cell in the serum-supplemented, attached cell culture.

15 *Viral production and cell culture in stirred and sparged bioreactor*

 In the first study the cells were successfully grown in an stirred bioreactor for viral production, and the oxygen and CO₂ were supplied by gas overlay in the headspace of a bioreactor. However, this method will limit the scale-up of the cell culture system because of its inefficient gas transfer. Therefore in the second study, to
20 test the feasibility of the scale up of the serum-free suspension culture and investigate the growth of cells and Ad-p53 production in a sparged bioreactor, pure oxygen and CO₂ gases were supplied by bubbling through the serum-free IS293 media supplemented with F-68 (0.1%) and heparin (100 mg/L).

25 Pure oxygen was bubbled through the liquid media to supply the dissolved oxygen to the cells and the supply of pure oxygen was controlled by a solenoid valve to keep the dissolved oxygen above 40%. For efficient oxygen supply while minimizing the damage to the cells a stainless steel sintered air diffuser, with a nominal pore size of which is approximately 0.22 micrometer, was used for the pure
30 oxygen delivery. The CO₂ gas was also supplied to the liquid media by bubbling from the same diffuser and tube as the pure oxygen to maintain the pH around 7.0.

For pH control Na_2CO_3 solution (106 g/L) was also hooked up to the bioreactor. Air was supplied to the head space of the bioreactor to keep a positive pressure inside the bioreactor. Other bioreactor configuration was the same as the first study.

5 Inoculum cells were developed from a frozen vial. One vial of frozen cells (1.0E+7 vc) was thawed into 50 mL media in a T-150 flask and subcultured 3 times in 200 mL media in 500 mL spinner flasks. 400 mL of inoculum cells grown in 2 of 500 mL spinner flasks were mixed with IS293 media with F-68 and heparin in a 10 L carboy to make 3.5 L cell suspension and it was transferred to the 5 L CelliGen
10 bioreactor.

 The initial cell density in the bioreactor was 3.0E+4 vc/mL. The initial cell density is lower than the first study. In the first study four of 500 mL spinner flasks were used as the inoculum. Even with the lower initial cell density the cells were
15 grown up to 1.8E+6 vc/mL on day 7 in the sparged environment and the viability was 98%. During the 7 days' growth, glucose concentration decreased from 5.4 g/L to 3.0 g/L and lactate increased from 0.3 g/L to 1.8 g/L.

 On day 7, when the cell density reached 1.8E+6 vc/mL, the cells in the
20 bioreactor were centrifuged down and resuspended in 3.5 L fresh serum-free IS293 media with F-68 and heparin in a 10 L carboy. The 293 cells were infected with 1.25E+11 pfu Ad-p53 and transferred to the CelliGen bioreactor. In the bioreactor, cell viability was 100% but the cell density was only 7.2E+5 vc/mL. There was a loss of cells during the media exchange operation. The viral titer in the media was
25 measured as 2.5E+10 HPLC vps/mL on day 2, 2.0E+10 on day 3, 2.8E+10 on day 4, 3.5E+10 on day 5 and 3.9E+10 HPLC vps/mL on day 6 of harvest. The first CelliGen bioreactor study with gas overlay produced 5.1E+10 HPLC vps/mL. The lower virus concentration in the second run was likely due to the lower cell density at the time of infection. Compared to the 7.2E+5 vc/mL in the second run, 2.1E+6 vc/mL was used
30 in the first run. Actually the per cell production of Ad-p53 in the second sparged CelliGen bioreactor is estimated to be 5.4E+4 vps/cell which is the highest per cell

production ever achieved so far. The per cell production in the first serum-free CellGen bioreactor without sparging and the serum-supplemented T-flask was $2.5E+4$ vps/cell and $3.5E+4$ vps/cell, respectively.

5 After the viral infection, the viability of the cells decreased from 100% to 13% on day 6 of harvest. During those 6 days after the infection the glucose concentration decreased from 5.0 g/L to 2.1 g/L and the lactate increased from 0.3 g/L to 2.9 g/L. During the entire period of operation about 20 mL of Na_2CO_3 (106 g/L) solution was consumed.

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The experimental result shows that it is technically and economically feasible to produce Ad-p53 in the sparged and stirred bioreactor. Scale-up and large-scale unit operation of sparged and stirred bioreactor are well established.

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* * *

All of the compositions and/or methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and/or methods and in the steps or in the sequence of steps of the method described herein without departing from the concept, spirit and scope of the invention. More specifically, it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the spirit, scope and concept of the invention as defined by the appended claims.

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References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

- 5
Aboud *et al.*, *Arch. Virol.*, 71:185-195, 1982.
Arap *et al.*, *Cancer Res.*, 55:1351-1354, 1995.
Bahnemann *et al.*, *Abs. Pap. ACS*, 180:5. 1980.
Baichwal and Sugden, *In: Gene transfer*, Kucherlapati R, ed., New York: Plenum
10 Press, pp. 117-148, 1986.
Benvenisty and Neshif, *Proc. Nat. Acad. Sci. USA*, 83:9551-9555, 1986.
Berg *et al.*, *BioTechniques*, 14(6):972-978, 1993.
Bett, *Proc. Natl. Acad. Sci. USA*, 91(19):8802-8806, 1994.
Bussemakers *et al.*, *Cancer Res.*, 52:2916-2922, 1992.
15 Caldas *et al.*, *Nat. Genet.*, 8:27-32, 1994.
Casey *et al.*, *Oncogene*, 6:1791-1797, 1991.
Chen and Okayama, *Mol. Cell Biol.*, 7:2745-2752, 1987.
Cheng *et al.*, *Cancer Res.*, 54:5547-5551, 1994.
Cheung *et al.*, *Biochem. J.*, 295:427-435, 1993c.
20 Cheung *et al.*, *J. Biol. Chem.*, 268:24303-24310, 1993a.
Cheung *et al.*, *J. Biol. Chem.*, 268:6139-6146, 1993b.
Coffin, *In: Virology*, Fields BN, Knipe DM, ed., New York: Raven Press, pp. 1437-
1500, 1990.
Coupar *et al.*, *Gene*, 68:1-10, 1988.

- Crooks *et al.*, *J. Chrom.*, 502: 59-68, 1990 .
- Dubensky *et al.*, *Proc. Nat. Acad. Sci. USA*, 81:7529-7533, 1984.
- Edelman and Crossin, *Annu. Rev. Biochem.*, 60:155-190, 1991
- Edelman, *Annu. Rev. Biochem.*, 54:135-169, 1985.
- 5 Fechheimer *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:8463-8467, 1987.
- Ferkol *et al.*, *FASEB J*, 7:1081-1091, 1993.
- Fraley *et al.*, *Proc. Natl. Acad. Sci. USA*, 76:3348-3352, 1979.
- Freedman *et al.*, WO 94/17178 (August 4, 1994).
- Frixen *et al.*, *J. Cell Biol.*, 113:173-185, 1991.
- 10 Garnier *et al.*, *Cytotechnol.*, 15:145-155, 1994.
- Ghosh and Bachhawat, *In: Liver diseases, targeted diagnosis and therapy using specific receptors and ligands*, Wu G, Wu C ed., New York: Marcel Dekker, pp. 87-104, 1991.
- Giancotti and Ruoslahti, *Cell*, 60:849-859, 1990.
- 15 Gilbert, "Adaptation of cells to serum free culture for production of adenovirus vectors and recombinant proteins," *Williamsburg BioProcessing Conference*, Nov. 18-21, 1996.
- Gopal, *Mol. Cell Biol.*, 5:1188-1190, 1985.
- Graham and Prevec, *In: Methods in Molecular Biology: Gene Transfer and*
- 20 *Expression Protocols 7*, Murray, E.J., (Eds.), Clifton, NJ: Humana Press, 109-128 and 205-225, 1991.
- Graham and Van Der Eb, *Virology*, 52:456-467, 1973.
- Graham *et al.*, *J. Gen. Virol.*, 36:59-74, 1977.

- Graham, J. *Gen. Virol.*, 68:93 7-940, 1987.
- Griffiths, In: *Animal Cell Biotechnology*, vol. 3, p179-220, (Eds., Spier, R.E. and Griffiths, J.B.), Academic Press, London., 1986
- Harland and Weintraub, *J. Cell Biol.*, 101:1094-1099, 1985.
- 5 Hay *et al.*, *Journal of Molecular Biology*, 175:493-510, 1984.
- Hearing and Shenk, *Journal of Molecular Biology*, 167:809-822, 1983.
- Hearing *et al.*, *Journal of Virology*, 67:2555-2558, 1987.
- Hermonat and Muzycska, *Proc. Nat. Acad. Sci. USA*, 81:6466-6470, 1984.
- Hollestein *et al.*, *Science*, 253:49-53 1991.
- 10 Hussussian *et al.*, *Nature Genetics*, 15-21, 1994.
- Huyghe *et al.*, *Human Gene Therapy*, 6:1403-1416, 1996.
- Jones and Shenk, *Cell*, 13:181-188, 1978.
- Kamb *et al.*, *Nature Genetics*, 8:22-26, 1994.
- Kamb *et al.*, *Science*, 267:436-440, 1994.
- 15 Kaneda *et al.*, *Science*, 243:375-378, 1989.
- Kato *et al.*, *J. Biol. Chem.*, 266:3361-3364, 1991.
- Klein *et al.*, *Nature*, 327:70-73, 1987.
- Larsson and Litwin, *Dev. Biol. Standard.*, 66:385-390, 1987.
- Leverro *et al.*, *Gene*, 101:195-202, 1991.
- 20 Lim, US Patent 4,352,883, October 5, 1982.
- Lin and Guidotti, *J. Biol. Chem.*, 264:14408-14414, 1989.
- Mann *et al.*, *Cell*, 33:153-159, 1983.
- Matsura *et al.*, *Brit. J. Cancer*, 66:1122-1130, 1992.

- McGrath *et al.*, *J. Virol.*, 25: 923-927, 1978.
- Mercer, *Critic. Rev. Eukar. Gene Express.* 2:251-263, 1992.
- Mizrahi, *Process Biochem.*, (August):9-12, 1983.
- Montenarh, *Crit. Rev. Oncogen*, 3:233-256, 1992.
- 5 Mori *et al.*, *Cancer Res.*, 54:3396-3397, 1994.
- Morris *et al.*, "Serum-free production of adenoviral vectors for gene therapy,"
Williamsburg BioProcessing Conference, Nov. 18-21, 1996.
- Myers, EPO 0273085
- Nicolas and Rubenstein, *In: Vectors: A survey of molecular cloning vectors and their*
10 *uses*, Rodriguez RL, Denhardt DT, ed., Stoneham: Butterworth, pp. 493-513,
1988.
- Nicolau and Sene, *Biochim. Biophys. Acta*, 721:185-190, 1982.
- Nicolau *et al.*, *Methods Enzymol.*, 149:157-176, 1987.
- Nilsson and Mosbach, *Dev. Biol. Standard.*, 66:183-193, 1987
- 15 Nobri *et al.*, *Nature (London)*, 368:753-756, 1995.
- O'Neil and Balkovic, *Bio/Technol.*, 11:173-178, 1993.
- Obrink, *BioEssays.*, 13:227-233, 1991.
- Odin and Obrink, *Exp. Cell Res.*, 171:1-15, 1987.
- Okamoto *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:11045-11049, 1994.
- 20 Orlow *et al.*, *Cancer Res.*, 54:2848-2851, 1994.
- Paskind *et al.*, *Virology*, 67:242-248, 1975.
- Perales *et al.*, *Proc. Natl. Acad. Sci. USA*, 91:4086-4090, 1994.
- Perrin *et al.*, *Vaccine*, 13(13):1244-1250, 1995.

- Petricciani, *Dev. Biol. Standard.*, 66:3-12, 1985.
- Phillips *et al.*, In: *Large Scale Mammalian Cell Culture* (Feder, J. and Tolbert, W. R., eds.), Academic Press, Orlando, FL, U.S.A., 1985.
- Potter *et al.*, *Proc. Nat. Acad. Sci. USA*, 81:7161-7165, 1984.
- 5 Renan, *Radiother. Oncol.*, 19:197-218, 1990.
- Ridgeway, In: *Vectors: A survey of molecular cloning vectors and their uses*, Rodriguez RL, Denhardt DT, ed., Stoneham: Butterworth, pp. 467-492, 1988.
- Rippe *et al.*, *Mol. Cell Biol.*, 10:689-695, 1990.
- Roux *et al.*, *Proc. Nat'l Acad. Sci. USA*, 86:9079-9083, 1989.
- 10 Serrano *et al.*, *Nature*, 366:704-707, 1993.
- Serrano *et al.*, *Science*, 267:249-252, 1995.
- Smith and Lee, *Analytical Biochem.*, 86: 252-263, 1978.
- Takahashi *et al.*, *Cancer Res.*, 52:2340-2342, 1992.
- Temin, In: *Gene transfer*, Kucherlapati R, ed., New York: Plenum Press, pp. 149-188,
- 15 1986.
- Tibbetts, *Cell*, 12:243-249, 1977.
- Tur-Kaspa *et al.*, *Mol. Cell Biol.*, 6:716-718, 1986.
- Umbas *et al.*, *Cancer Res.*, 52:5104-5109, 1992.
- van Wezel, *Nature*, 216:64-65, 1967.
- 20 Wagner *et al.*, *Proc. Natl. Acad. Sci.*, 87(9):3410-3414, 1990.
- Wagner *et al.*, *Science*, 260:1510-1513, 1993.
- Wang *et al.*, In: *Animal Cell Technology: Basic & Applied Aspects*, S. Kaminogawa *et al.*, (eds), vol. 5, pp463-469, Kluwer Academic Publishers, Netherlands, 1993.

Wang *et al.*, *Cytotechnology*, 9:41-49, 1992.

Wang *et al.*, *Proceeding of the Japanese Society for Animal Cell Technology*, 1994.

Watt *et al.*, *Proc. Natl Acad. Sci.*, 83(2):3166-3170, 1986.

Weinberg, *Science*, 254:1138-1146, 1991.

5 Wong *et al.*, *Gene*, 10:87-94, 1980.

Wu and Wu, *J. Biol. Chem.*, 262:4429-4432, 1987.

Wu and Wu, *Adv. Drug Delivery Rev.*, 12:159-167, 1993.

Wu and Wu, *Biochemistry*, 27:887-892, 1988.

Yang *et al.*, *Proc. Natl Acad. Sci. USA*, 87:9568-9572, 1990.

CLAIMS:

1. A method for producing an adenovirus comprising:
 - a) growing host cells in media at a low perfusion rate;
 - 5 b) infecting said host cells with an adenovirus;
 - c) harvesting and lysing said host cells to produce a crude cell lysate;
 - d) concentrating said crude cell lysate;
 - e) exchanging buffer of crude cell lysate; and
 - 10 f) reducing the concentration of contaminating nucleic acids in said crude cell lysate.
2. The method of claim 1, further comprising isolating an adenoviral particle from said cell lysate using chromatography.
- 15 3. The method of claim 1, wherein the glucose concentration in said media is maintained between about 0.7 and about 1.7g/L.
4. The method of claim 1, wherein said exchanging buffer involves a diafiltration
20 step.
5. The method of claim 1, wherein said adenovirus comprises an adenoviral vector encoding an exogenous gene construct.

6. The method of claim 5, wherein said gene construct is operatively linked to a promoter.
- 5 7. The method of claim 6, wherein said promoter is SV40 IE, RSV LTR, β -actin, CMV IE, adenovirus major late, polyoma F9-1, or tyrosinase.
8. The method of claim 1, wherein said adenovirus is a replication-incompetent adenovirus.
- 10 9. The method of claim 8, wherein the adenovirus is lacking at least a portion of the E1-region.
- 15 10. The method of claim 9, wherein the adenovirus is lacking at least a portion of the E1A and/or E1B region.
11. The method of claim 1, wherein said host cells are capable of complementing replication.
- 20 12. The method of claim 1, wherein said host cells are 293 cells.
13. The method of claim 5, wherein said exogenous gene construct encodes a therapeutic gene.

14. The method of claim 13, wherein said therapeutic gene encodes antisense *ras*, antisense *myc*, antisense *raf*, antisense *erb*, antisense *src*, antisense *fms*, antisense *jun*, antisense *trk*, antisense *ret*, antisense *gsp*, antisense *hst*,
5 antisense *bcl* antisense *abl*, Rb, CFTR, p16, p21, p27, p57, p73, C-CAM, APC, CTS-1, *zac1*, scFV *ras*, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, MMAC1, FCC, MCC, BRCA2, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, GM-CSF G-CSF, thymidine kinase or p53.
- 10
15. The method of claim 14, wherein said therapeutic gene encodes p53.
16. The method of claim 1, wherein said cells are harvested and lysed *ex situ* using a hypotonic solution, hypertonic solution, freeze-thaw, sonication, impinging
15 jet, microfluidization or a detergent.
17. The method of claim 1, wherein said cells are harvested and lysed *in situ* using a hypotonic solution, hypertonic solution, or a detergent.
- 20 18. The method of claim 17, wherein said cells are lysed and harvested using detergent.

19. The method of claim 18, wherein said detergent is Thesit[®], NP-40[®], Tween-20[®], Brij-58[®], Triton X[®]-100 or octyl glucoside.
20. The method of claim 1, wherein said lysis is achieved through autolysis of infected cells.
21. The method of claim 1, wherein said cell lysate is treated with Benzonase[®], or Pulmozyme[®].
22. The method of claim 2, wherein said isolating consists essentially of a single chromatography step.
23. The method of claim 22, wherein said chromatography step is ion exchange chromatography.
24. The method of claim 23, wherein said ion exchange chromatography is anion exchange chromatography.
25. The method of claim 24, wherein said anion exchange chromatography utilizes DEAE, TMAE, QAE, or PEI.
26. The method of claim 24, wherein said anion exchange chromatography utilizes Toyopearl Super Q 650M, MonoQ, Source Q or Fractogel TMAE.

27. The method of claim 24, wherein said ion exchange chromatography is carried out at a pH range of between about 7.0 and about 10.0.
- 5 28. The method of claim 1, further comprising a concentration step employing membrane filtration.
29. The method of claim, 28, wherein said filtration is tangential flow filtration.
- 10 30. The method of claim, 28, wherein said filtration utilizes a 100 to 300K NMWC, regenerated cellulose, or polyether sulfone membrane.
31. An adenovirus produced according to a process comprising the steps of:
- a) growing host cells in media at a low perfusion rate;
 - 15 b) infecting said host cells with an adenovirus;
 - c) harvesting and lysing said host cells to produce a crude cell lysate;
 - d) concentrating said crude cell lysate;
 - e) exchanging buffer of crude cell lysate; and
 - 20 f) reducing the concentration of contaminating nucleic acids in said crude cell lysate.

32. The adenovirus of claim 31, wherein adenovirus comprises an adenoviral vector encoding an exogenous gene construct.
33. The adenovirus of claim 31, wherein said gene construct is operatively linked to a promoter.
34. The adenovirus of claim 31, wherein said adenovirus is a replication-incompetent adenovirus.
35. The adenovirus of claim 34, wherein said adenovirus is lacking at least a portion of the E1-region.
36. The adenovirus of claim 31, wherein the adenovirus is lacking at least a portion of the E1A and/or E1B region.
37. The adenovirus of claim 31, wherein said host cells are capable of complementing replication.
38. The adenovirus of claim 31, wherein said host cells are 293 cells.
39. The adenovirus of claim 31, wherein said exogenous gene construct encodes a therapeutic gene.

40. The adenovirus of claim 39, wherein said therapeutic gene encodes antisense *ras*, antisense *myc*, antisense *raf*, antisense *erb*, antisense *src*, antisense *fms*, antisense *jun*, antisense *trk*, antisense *ret*, antisense *gsp*, antisense *hst*, antisense *bcl* antisense *abl*, Rb, CFTR, p16, p21, p27, p57, p73, C-CAM, APC, CTS-1, *zac1*, scFV *ras*, DCC, NF-1, NF-2, WT-1, MEN-I, MEN-II, BRCA1, VHL, MMAC1, FCC, MCC, BRCA2, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11 IL-12, GM-CSF G-CSF, thymidine kinase or p53.
- 10 41. The adenovirus of claim 40, wherein said therapeutic gene is p53.
42. The adenovirus of claim 33, wherein said promoter is SV40 IE, RSV LTR, β -actin or CMV IE, adenovirus major late, polyoma F9-1, or tyrosinase.
- 15 43. A method for the purification of an adenovirus comprising:
- a) growing host cells;
 - b) infecting said host cells with an adenovirus;
 - c) harvesting and lysing said host cells by contacting said cells with a detergent to produce a crude cell lysate;
 - 20 d) concentrating said crude cell lysate;
 - e) exchanging buffer of crude cell lysate; and
 - f) reducing the concentration of contaminating nucleic acids in said crude cell lysate.

44. The method of claim 43, further comprising isolating an adenoviral particle from said lysate using chromatography.
- 5 45. The method of claim 43, wherein said host cells are grown in media wherein a glucose concentration is maintained between about 0.7 and about 1.7g/L.
46. The method of claim 43, wherein said exchanging buffer involves a diafiltration step.
- 10 47. The method of claim 43, wherein said detergent is Thesit[®], NP-40[®], Tween-20[®], Brij-58[®], Triton X-100[®] or octyl glucoside.
48. The method of claim 47, wherein said detergent is present in the lysis solution at a concentration of about 1% (w/v).
- 15 49. The method of claim 43, wherein said isolating consists essentially of a single chromatography step.
- 20 50. The method of claim 44, wherein said chromatography step is ion exchange chromatography.
51. An adenovirus produced according to a process comprising the steps of:

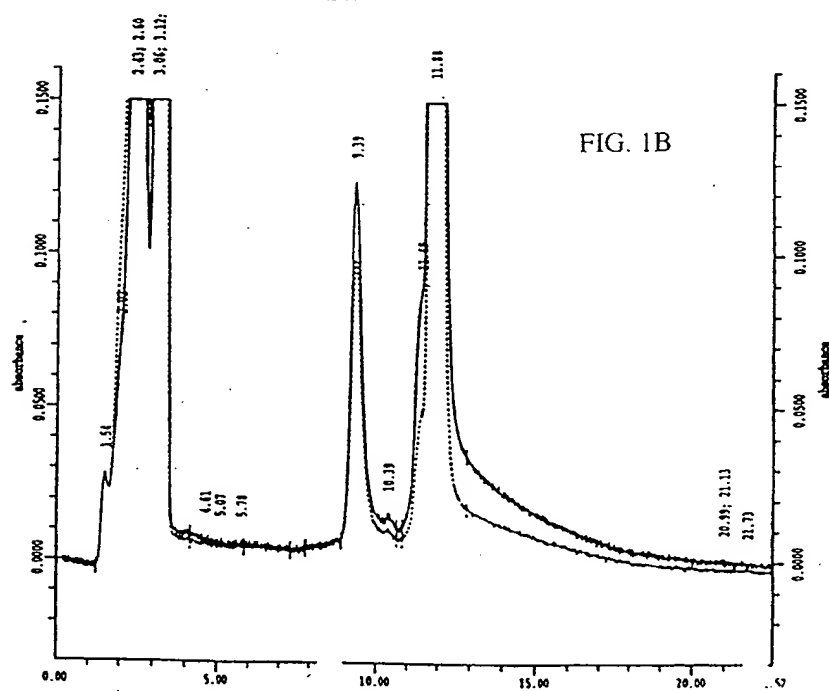
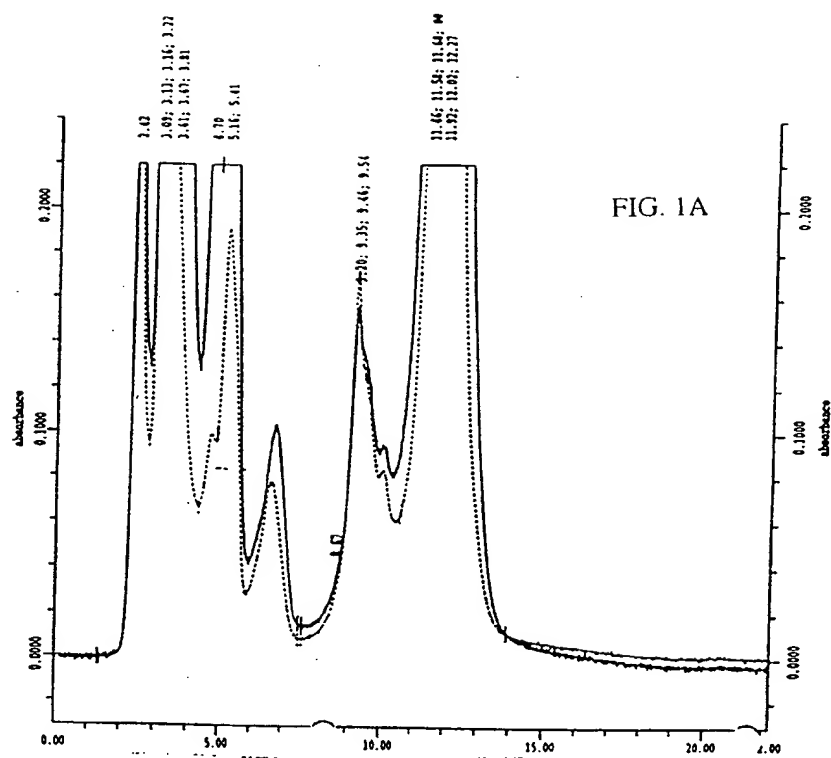
- a) growing host cells;
 - b) infecting said host cells with an adenovirus;
 - c) harvesting and lysing said host cells by contacting said cells with a detergent to produce a crude cell lysate;
 - 5 d) concentrating said crude cell lysate;
 - e) exchanging buffer of crude cell lysate; and
 - f) reducing the concentration of contaminating nucleic acids in said crude cell lysate.
- 10 52. A method for the purification of an adenovirus comprising:
- a) growing host cells in serum-free media;
 - b) infecting said host cells with an adenovirus;
 - c) harvesting and lysing said host cells to produce a crude cell lysate;
 - 15 d) concentrating said crude cell lysate;
 - e) exchanging buffer of crude cell lysate; and
 - f) reducing the concentration of contaminating nucleic acids in said crude cell lysate.
- 20 53. The method of claim 52, wherein said host cells are adapted for growth in serum-free media.

54. The method of claim 52, wherein said cells are grown as a cell suspension culture.
55. The method of claim 52, wherein said cells are grown as an anchorage-
5 dependent culture.
56. The method of claim 53, wherein said adaptation for growth in serum-free media comprises a sequential decrease in the fetal bovine serum content of the growth media.
- 10 57. The method of claim 53, wherein said serum-free media comprises a fetal bovine serum content of less than 0.03% v/v.
58. The method of claim 52, further comprising isolating an adenoviral particle
15 from said lysate using chromatography.
59. The method of claim 52, wherein said lysis is achieved through autolysis of infected cells.
- 20 60. The method of claim 52, wherein said exchanging buffer involves a diafiltration step.

61. The method of claim 52, wherein said detergent is Thesit[®], NP-40[®], Tween-20[®], Brij-58[®], Triton X-100[®] or octyl glucoside.
62. The method of claim 52, wherein said detergent is present in the lysis solution
5 at a concentration of about 1% (w/v).
63. The method of claim 52, wherein said isolating consists essentially of a single chromatography step.
- 10 64. The method of claim 58, wherein said chromatography step is ion exchange chromatography.
65. An adenovirus produced according to a process comprising the steps of:
- 15 a) growing host cells in serum-free media;
- b) infecting said host cells with an adenovirus;
- c) harvesting and lysing said host cells to produce a crude cell lysate;
- d) concentrating said crude cell lysate;
- e) exchanging buffer of crude cell lysate; and
- 20 f) reducing the concentration of contaminating nucleic acids in said crude cell lysate.
66. A 293 host cell adapted for growth in serum-free media.

67. The cell of claim 66, wherein said cell is adapted for growth in suspension culture.
- 5 68. The cell of claim 66, wherein the cell is deposited with the ATCC and is designated as a IT293SF cell.
69. The cell of claim 66, wherein said adaptation for growth in serum-free media comprises a sequential decrease in the fetal bovine serum content of the growth media.
- 10

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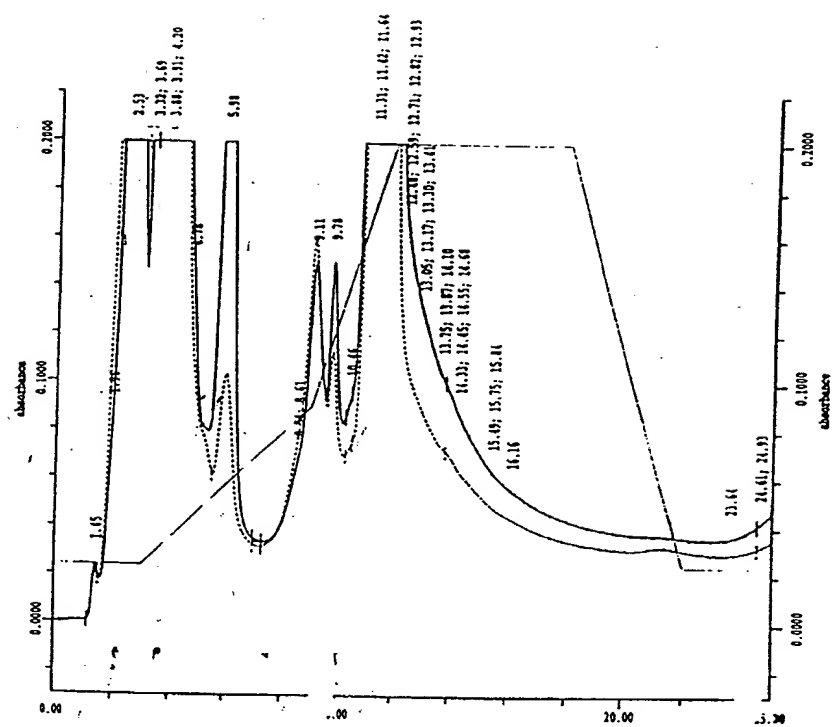


FIG. 2

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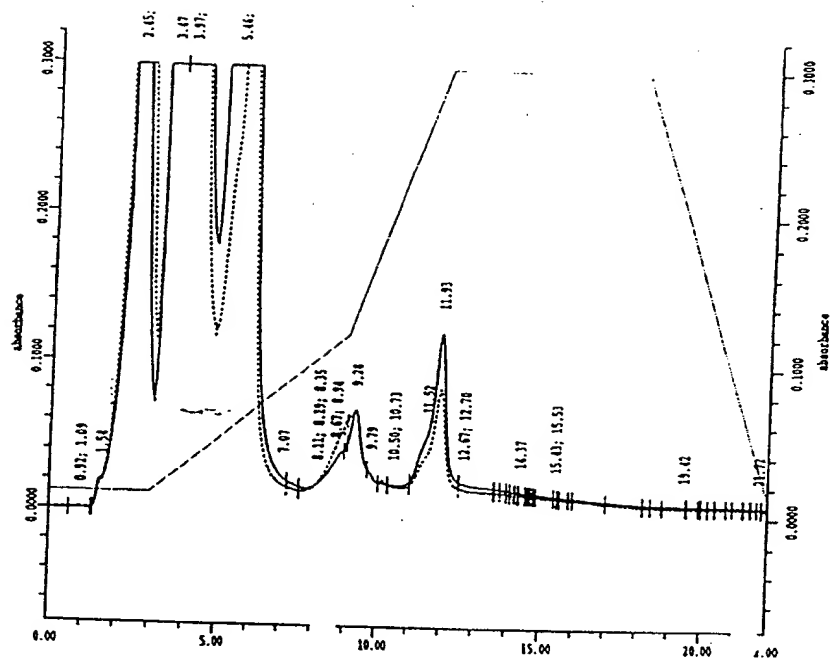


FIG. 3C

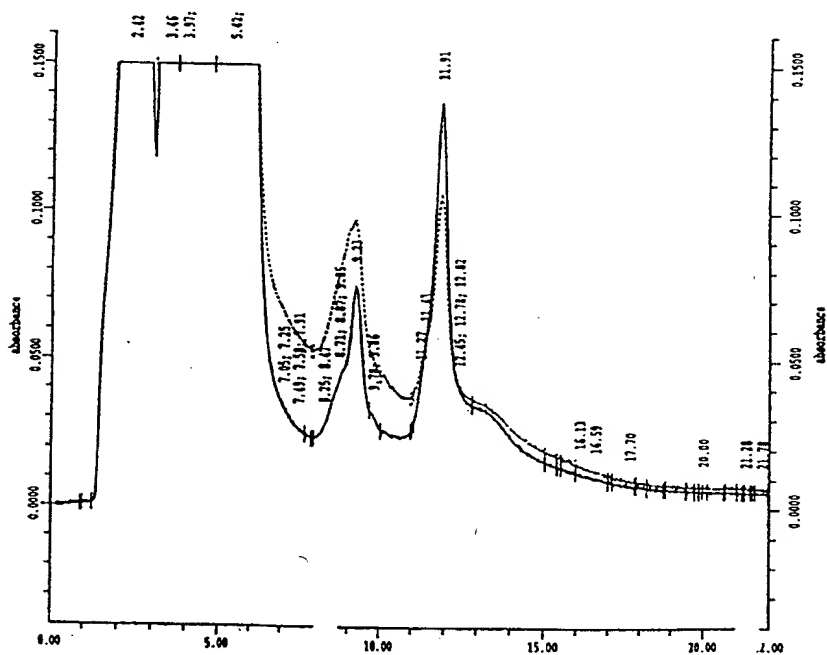


FIG. 3D

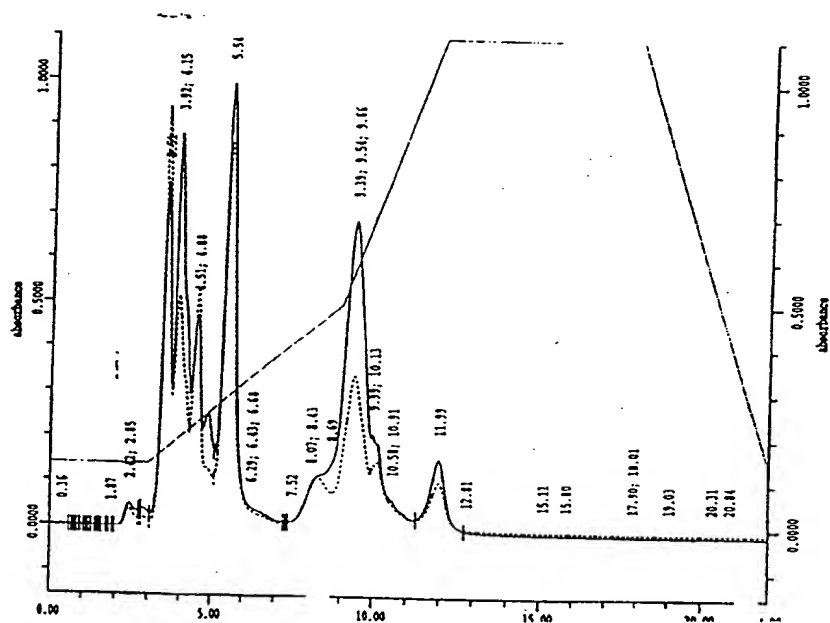


FIG.3E

7/32

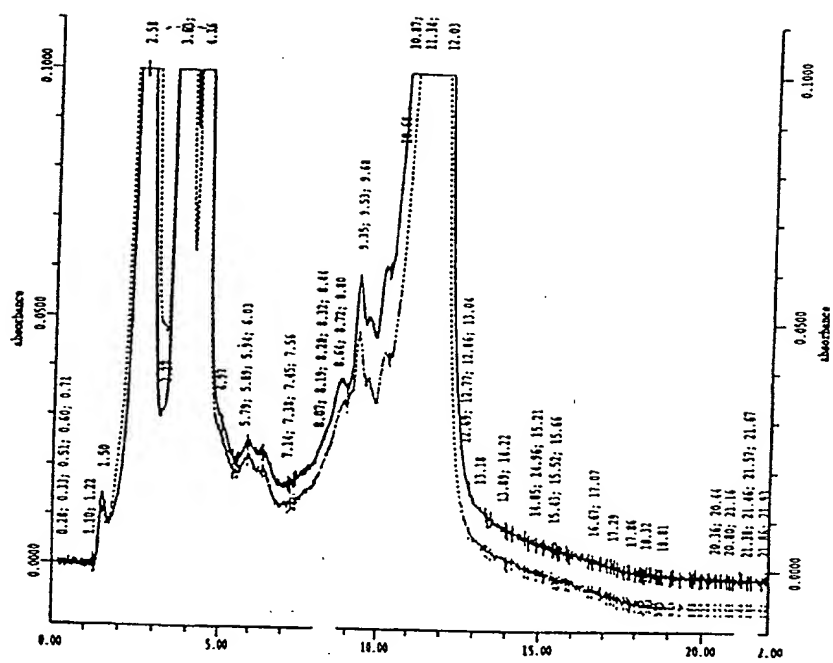


FIG.5

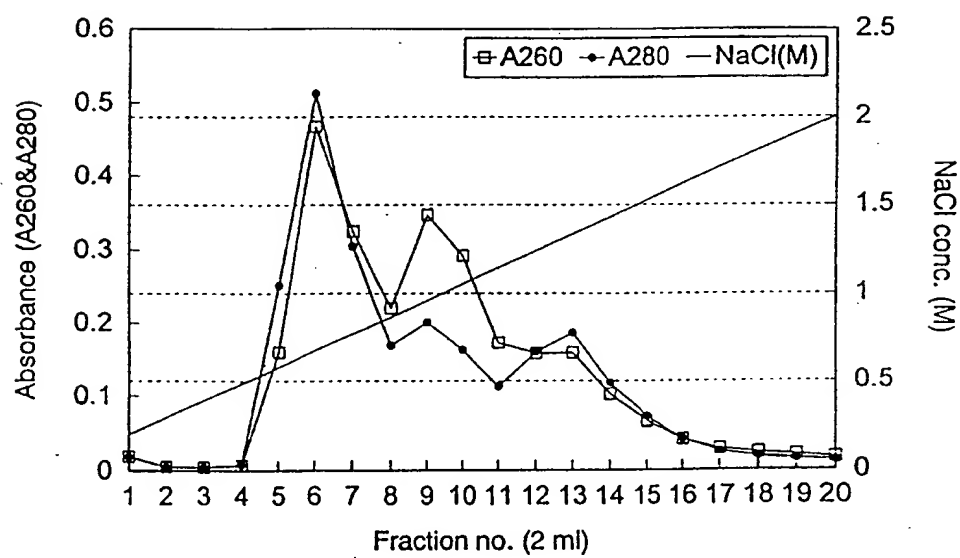


FIG.6

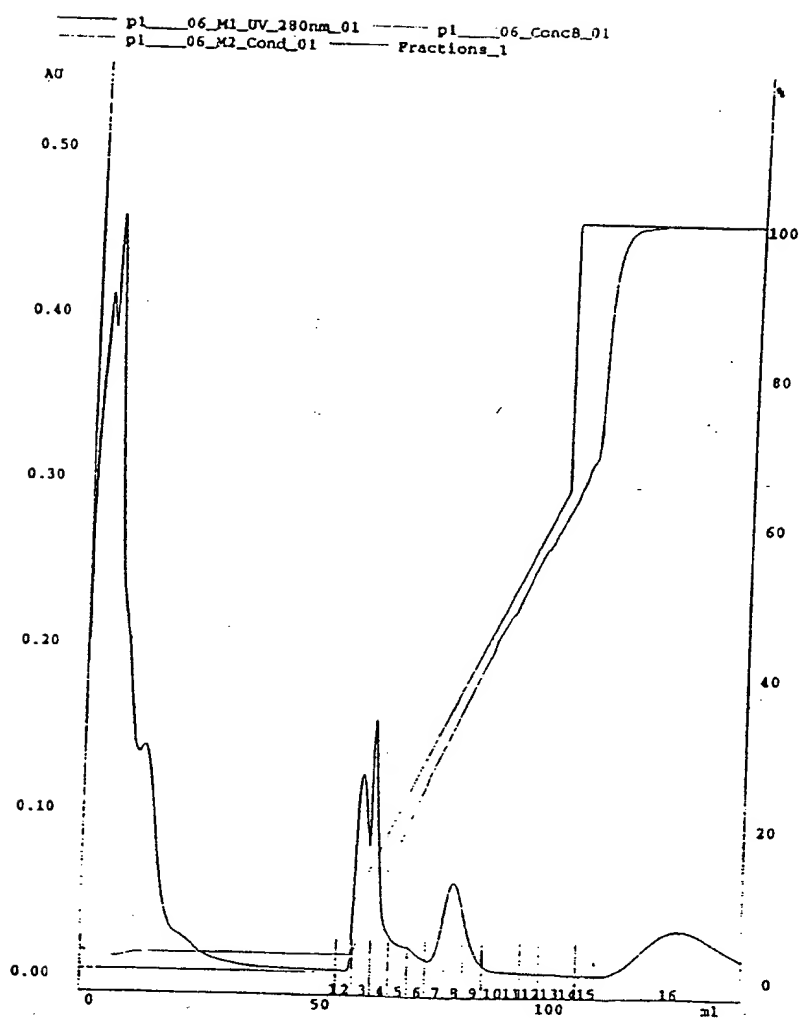
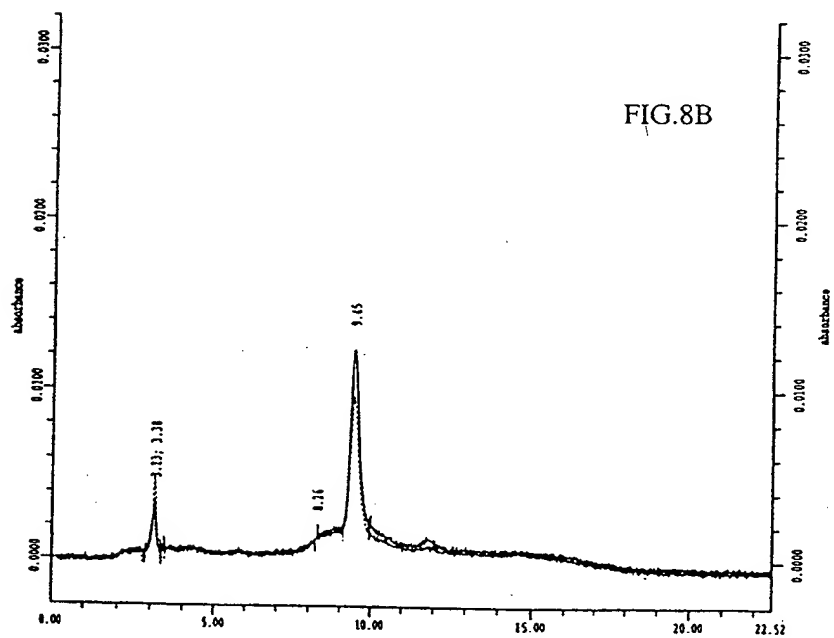
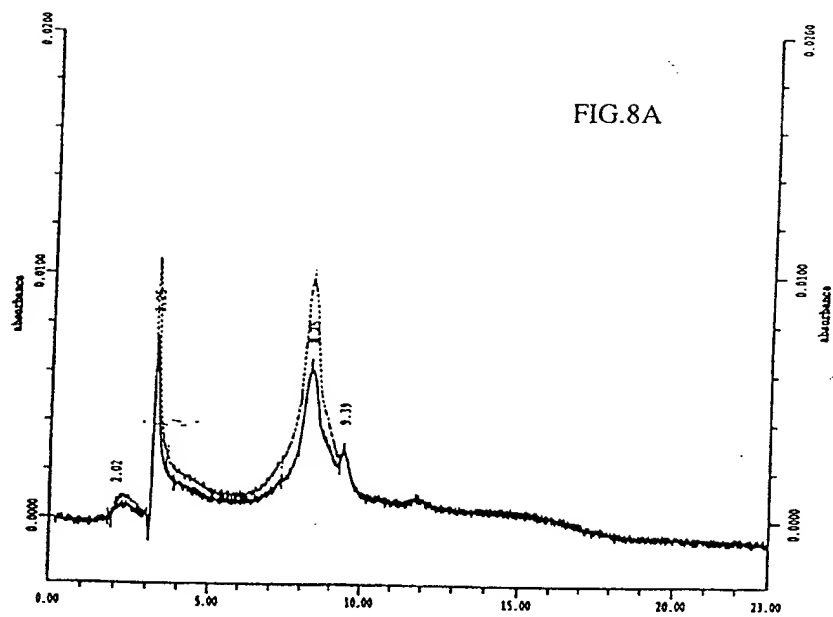
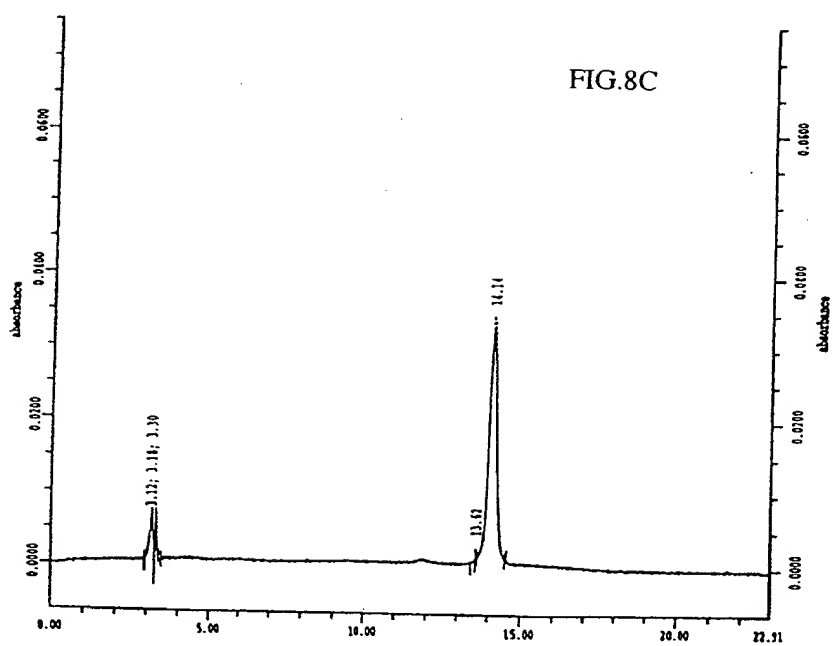


FIG.7

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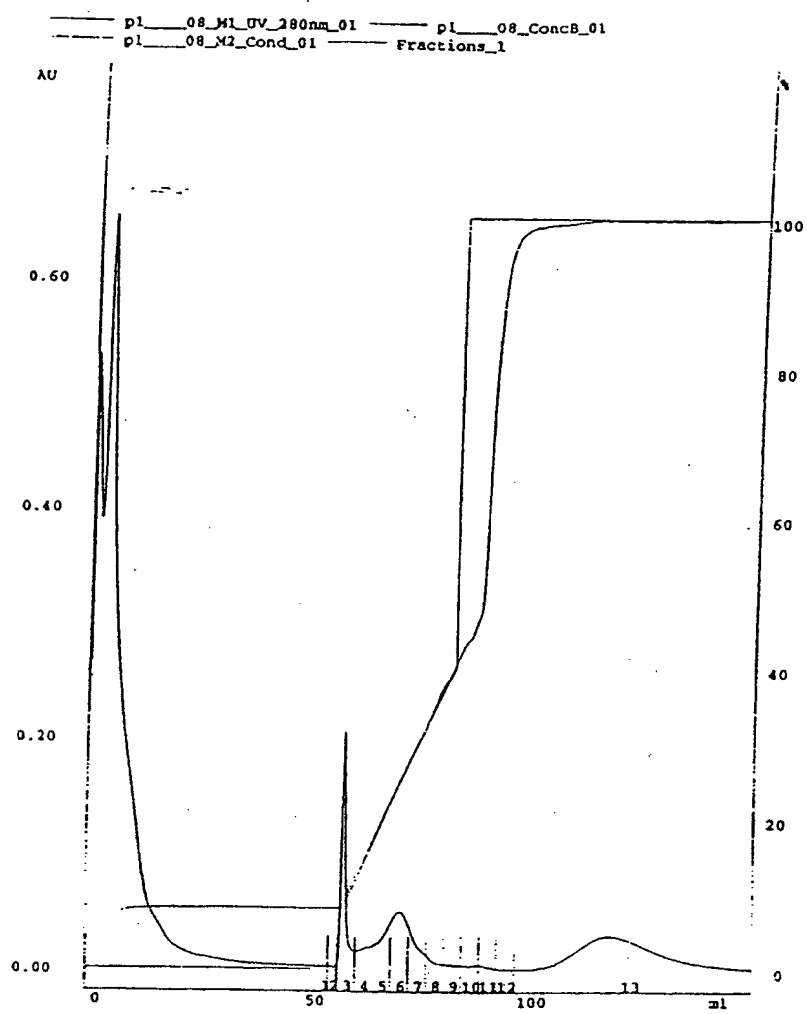
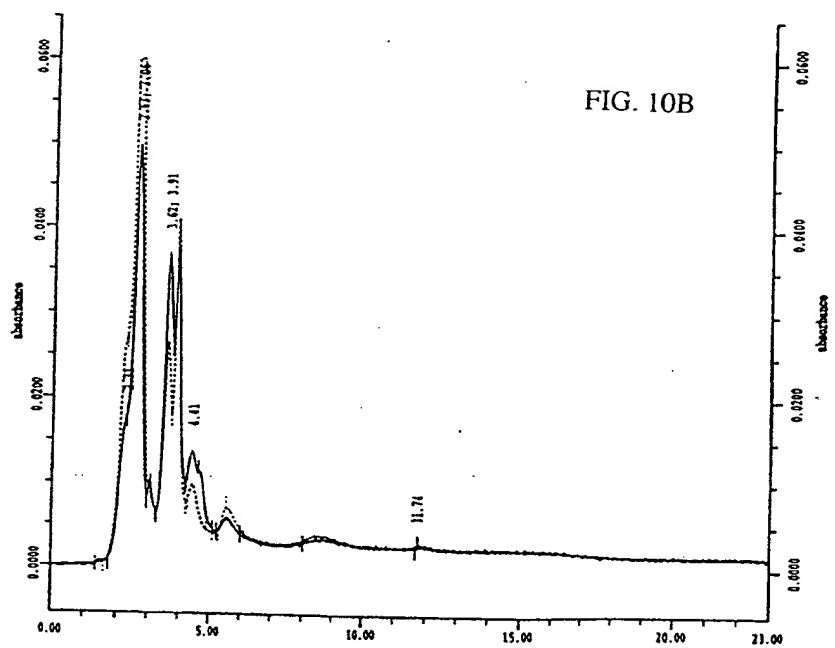
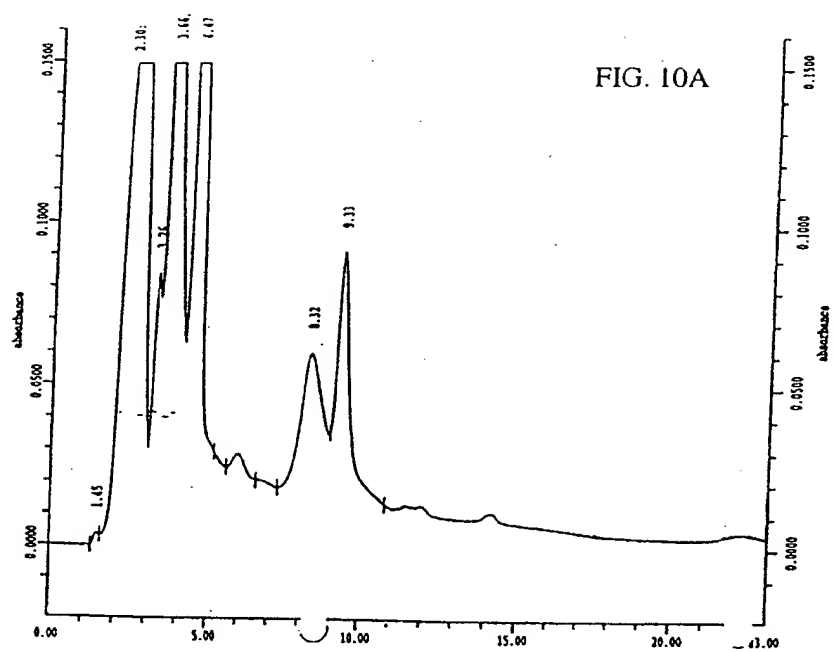
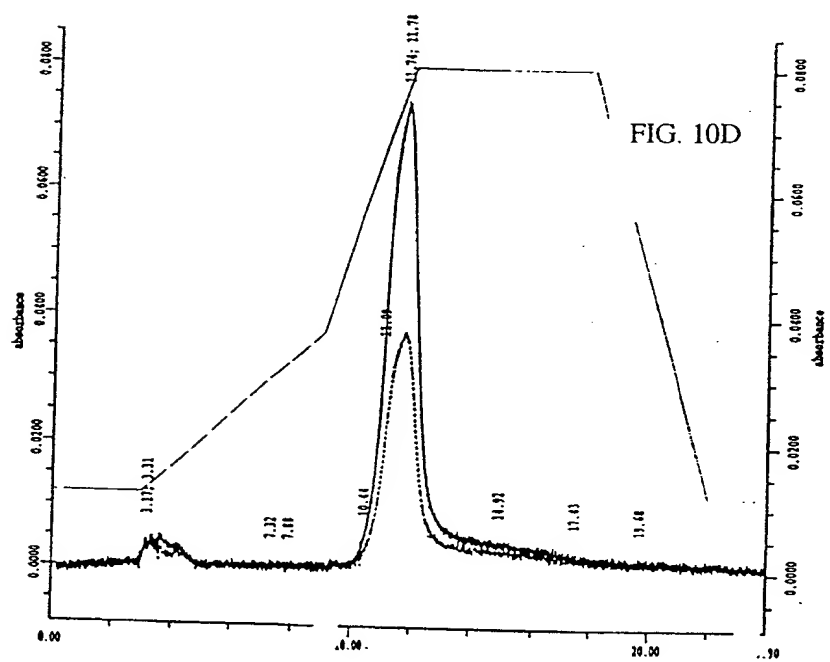
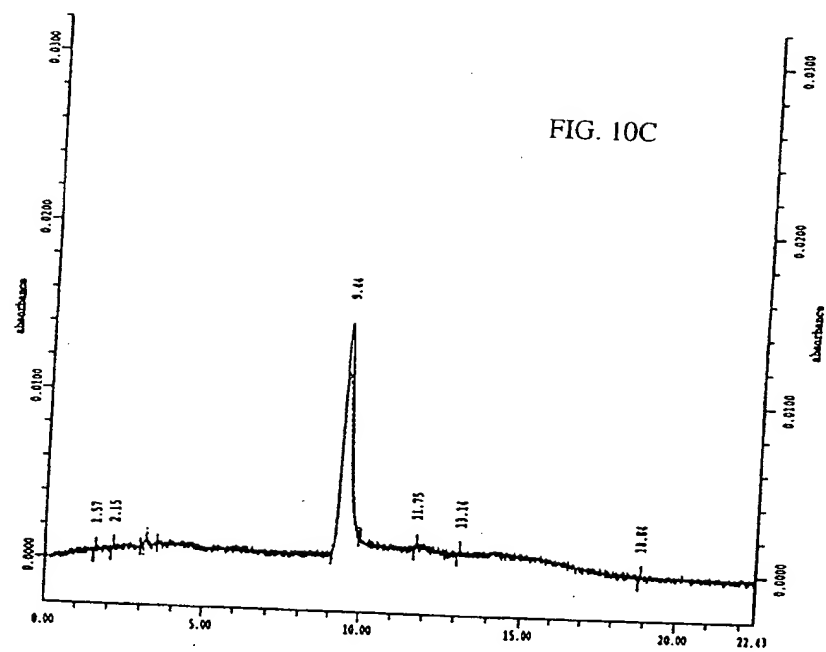


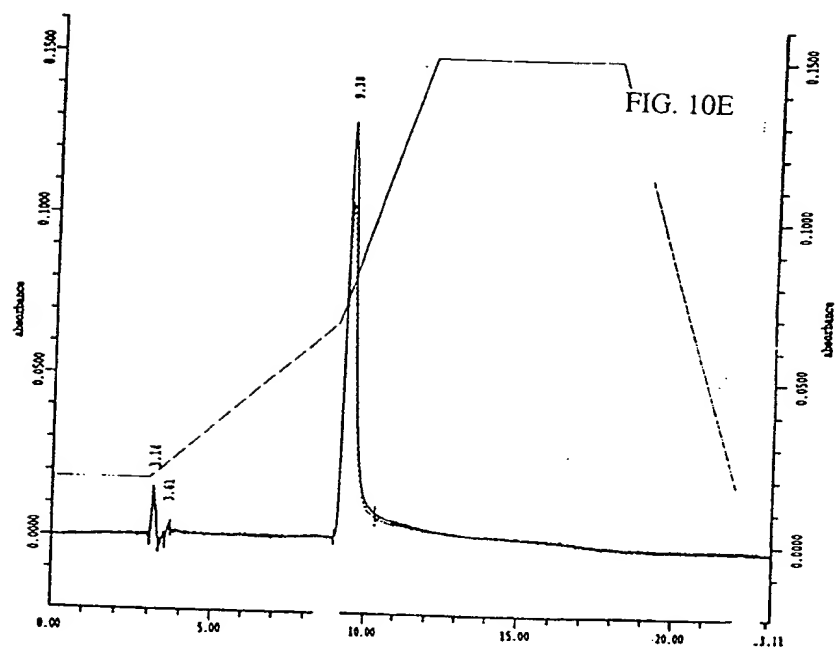
FIG. 9

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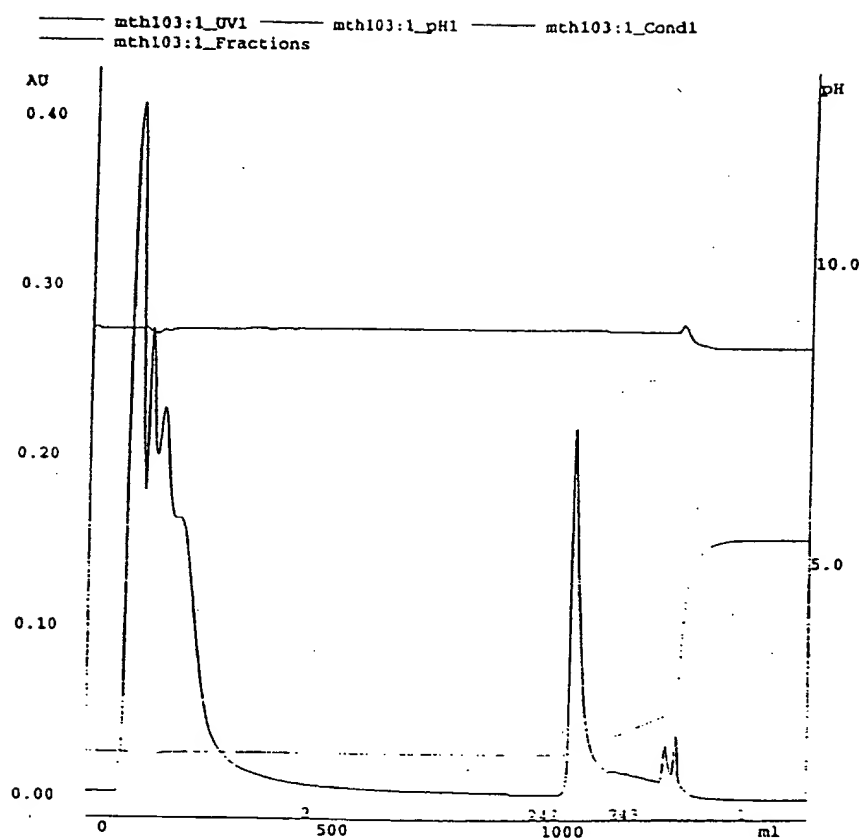


FIG. 11

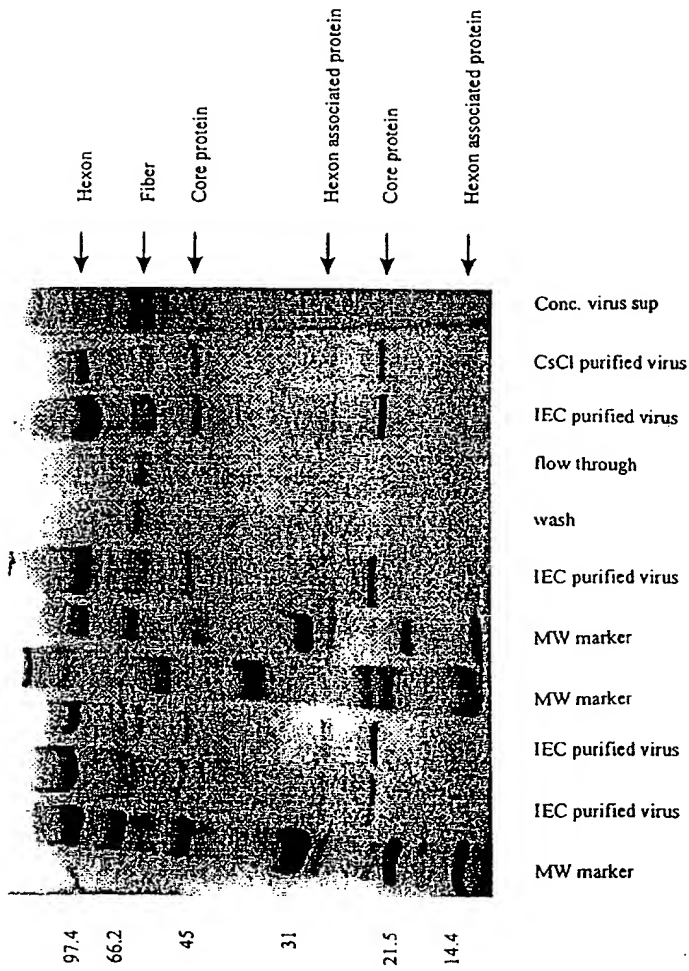


FIG. 12

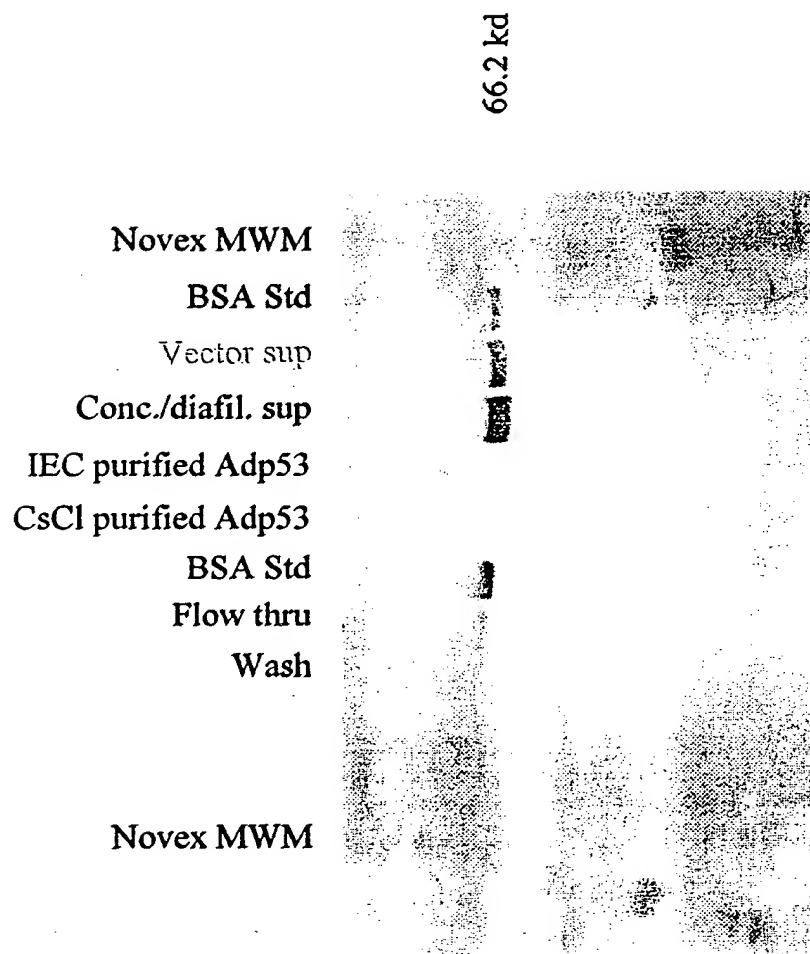


FIG. 13

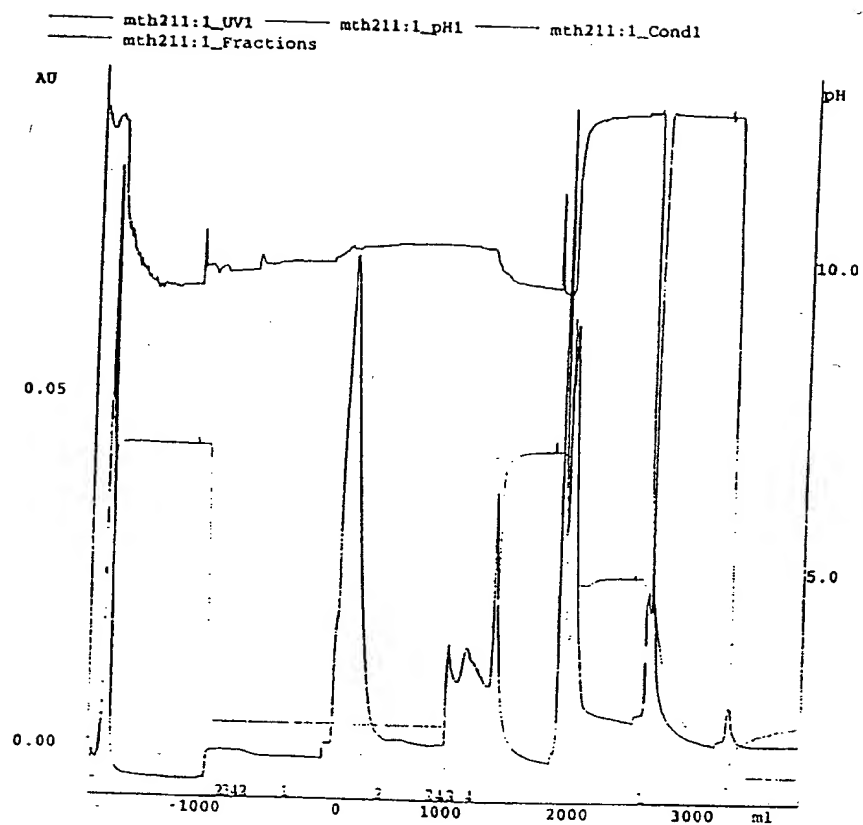


FIG. 14

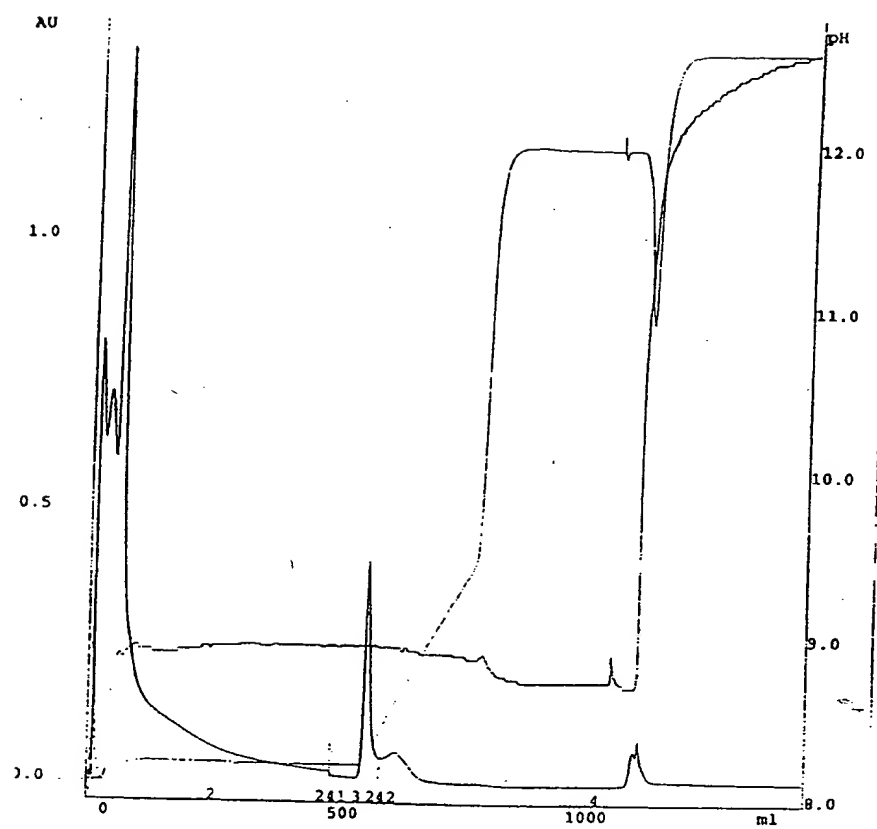
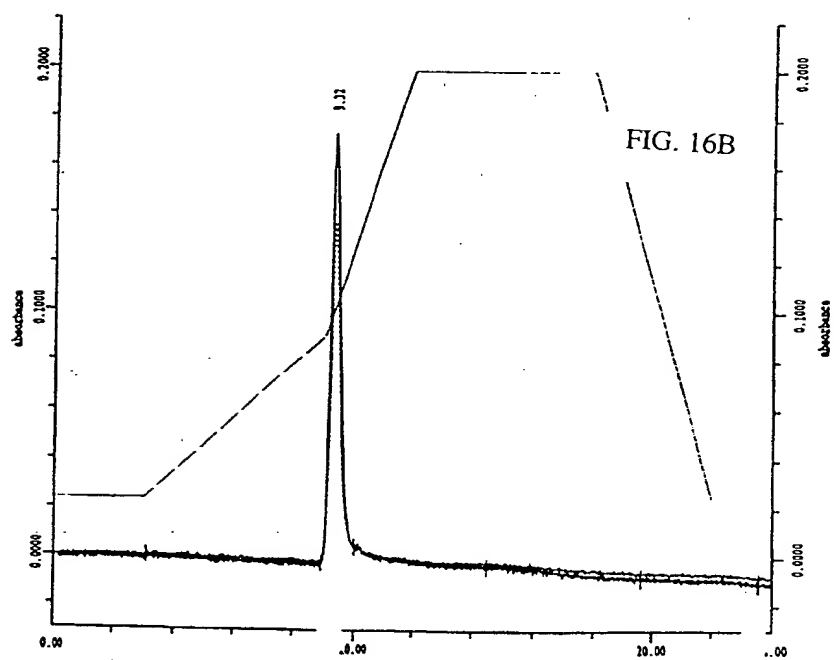
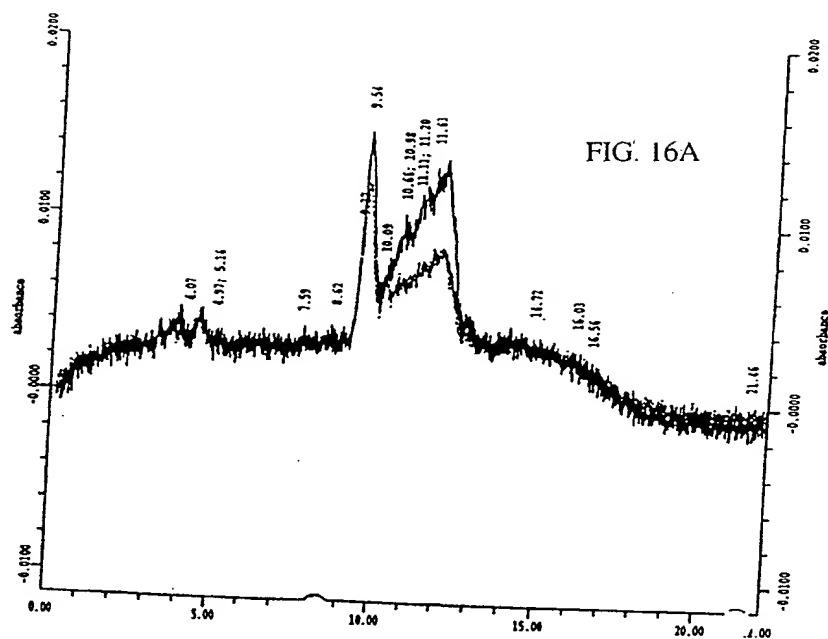


FIG. 15



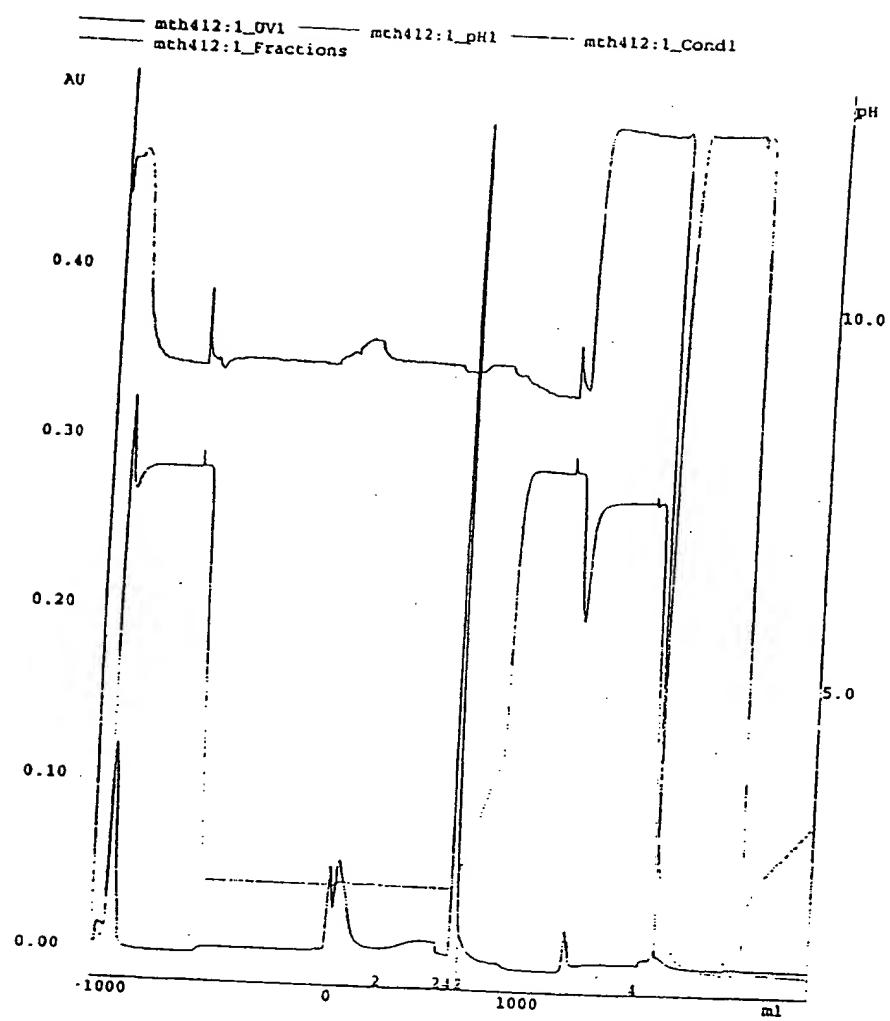


FIG. 17

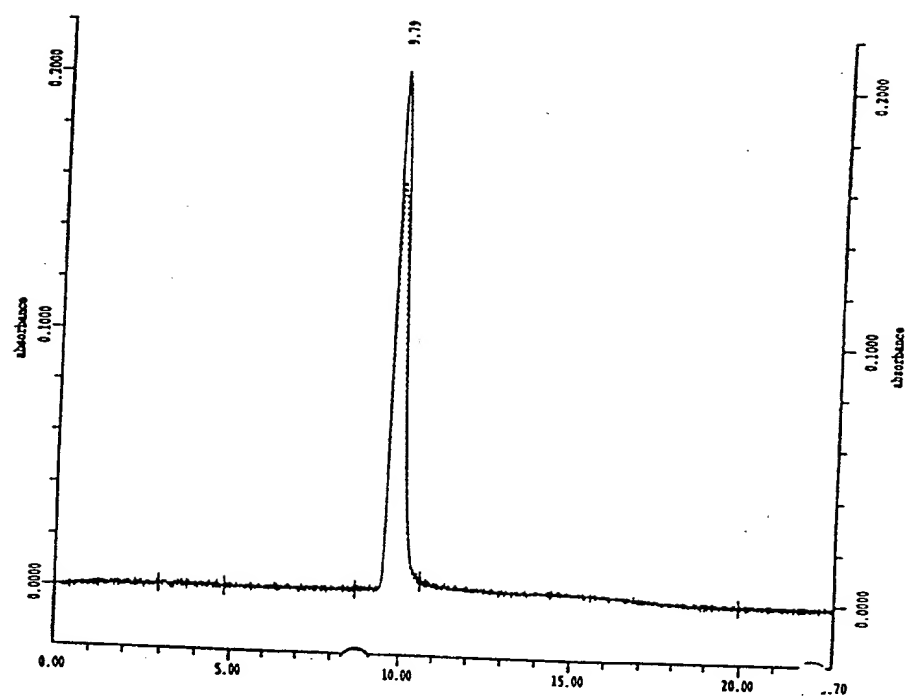
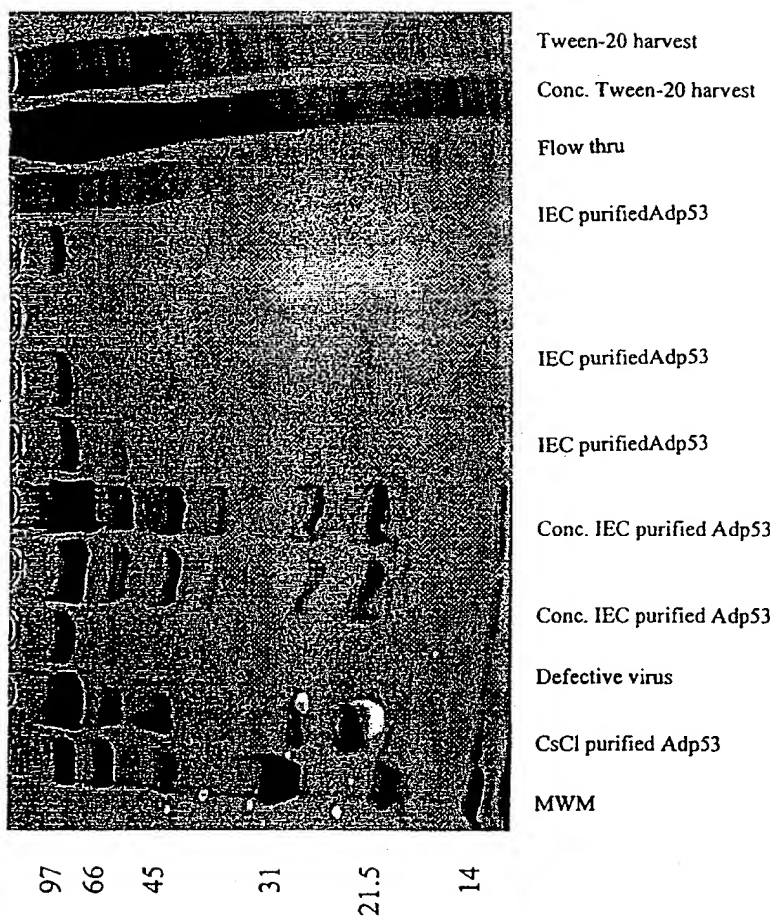


FIG. 18

FIG. 19A



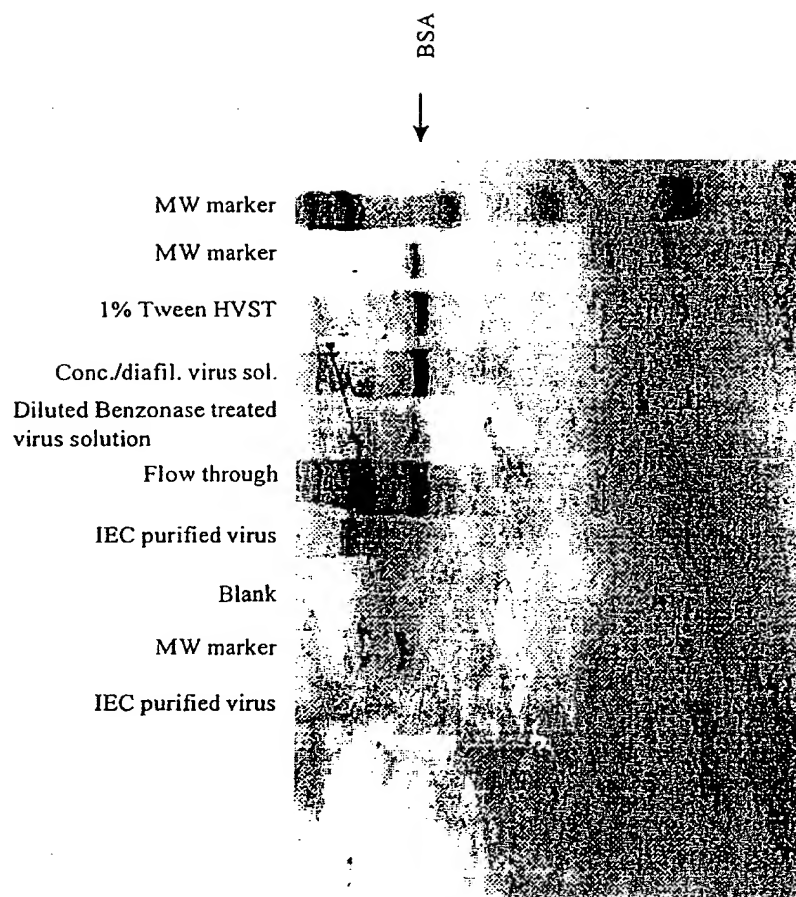


FIG. 19B

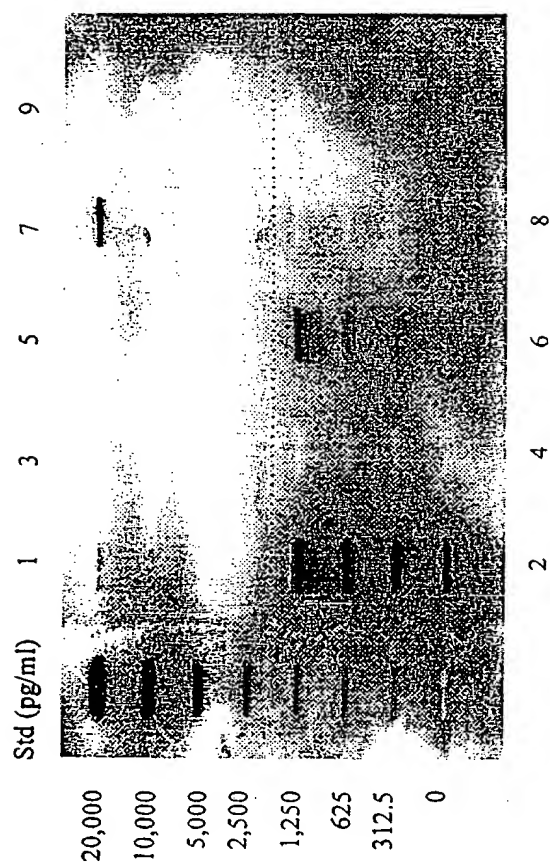
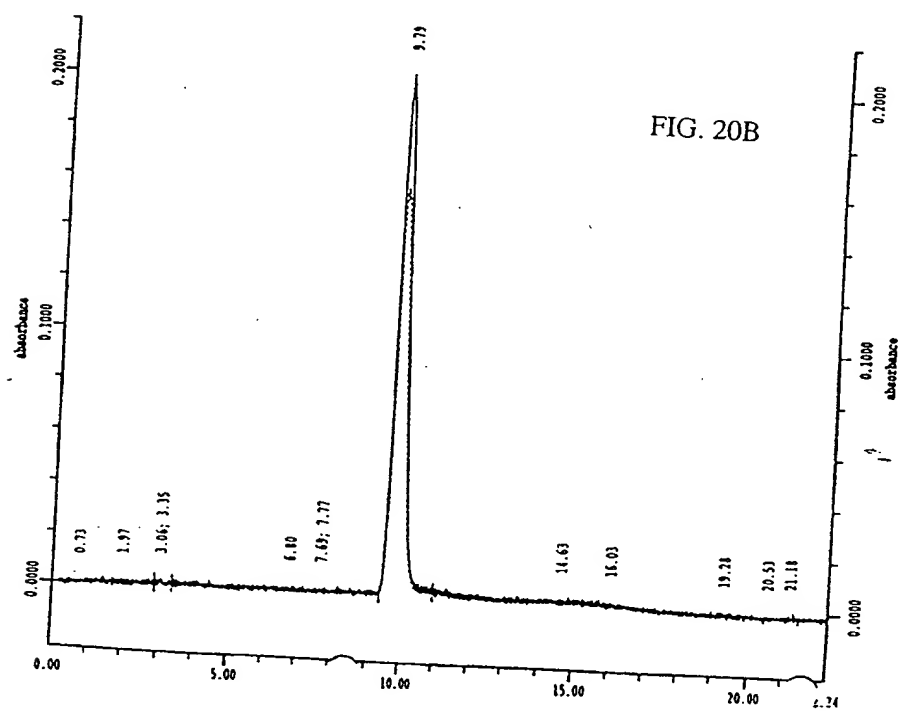
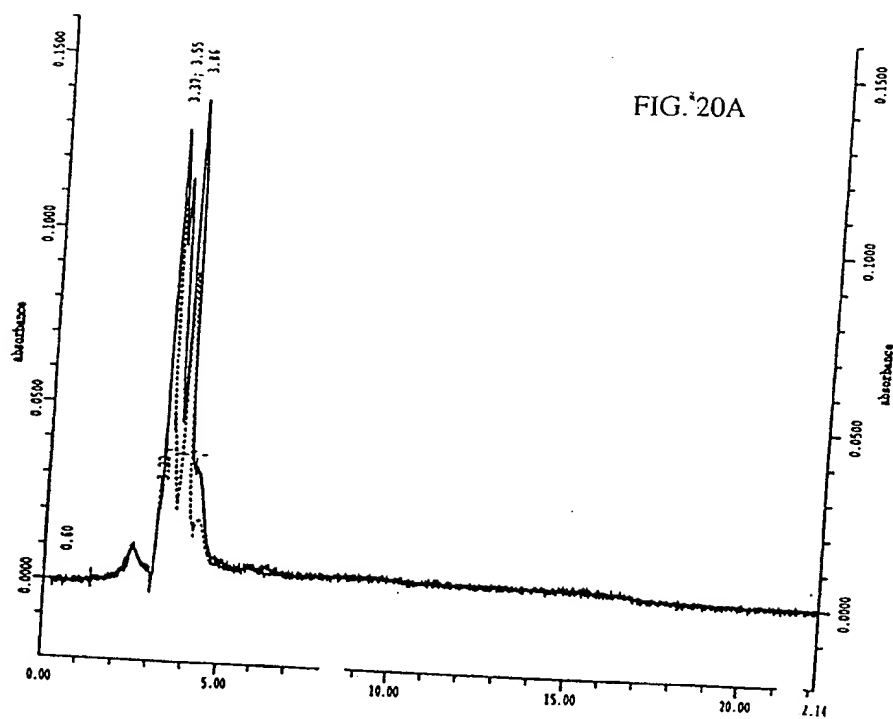
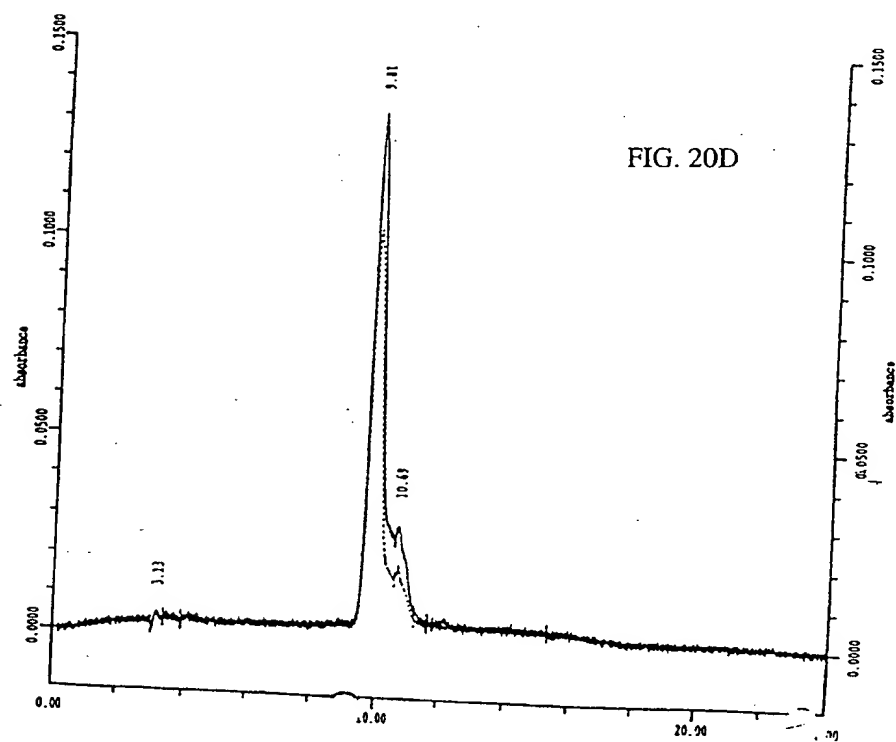
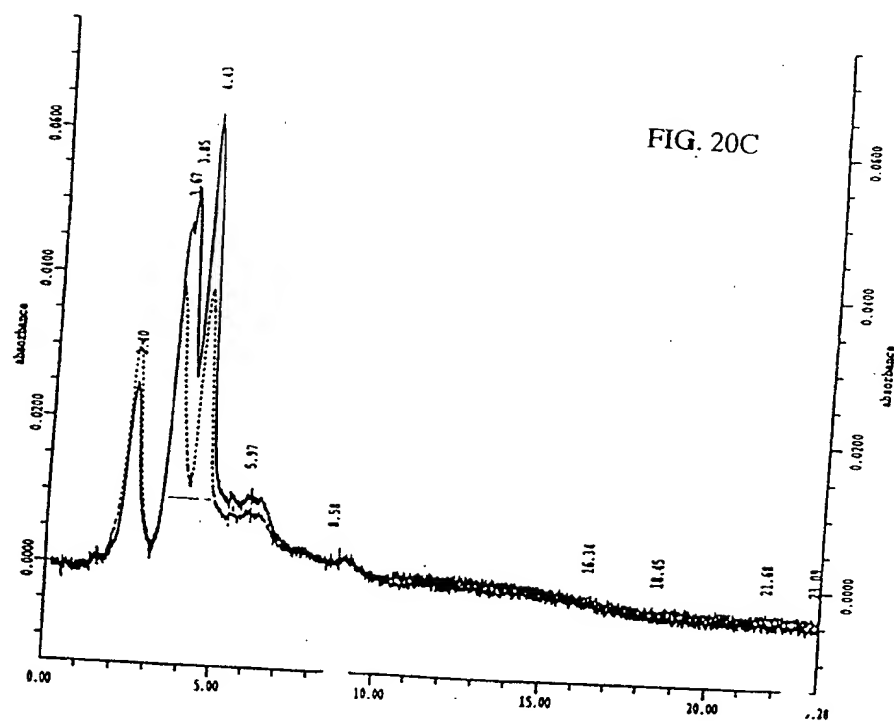


FIG. 19C

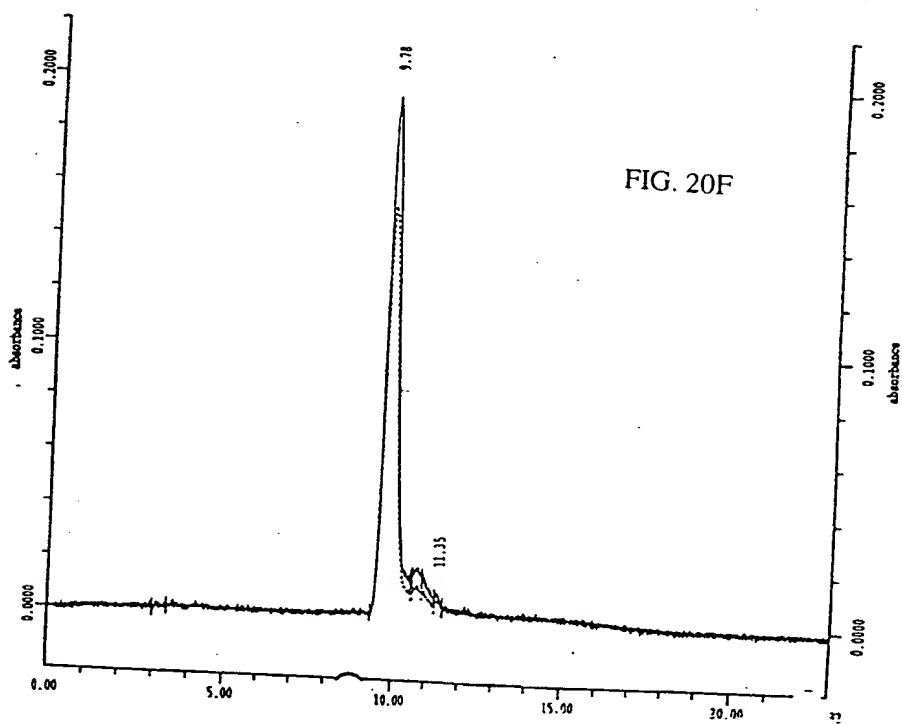
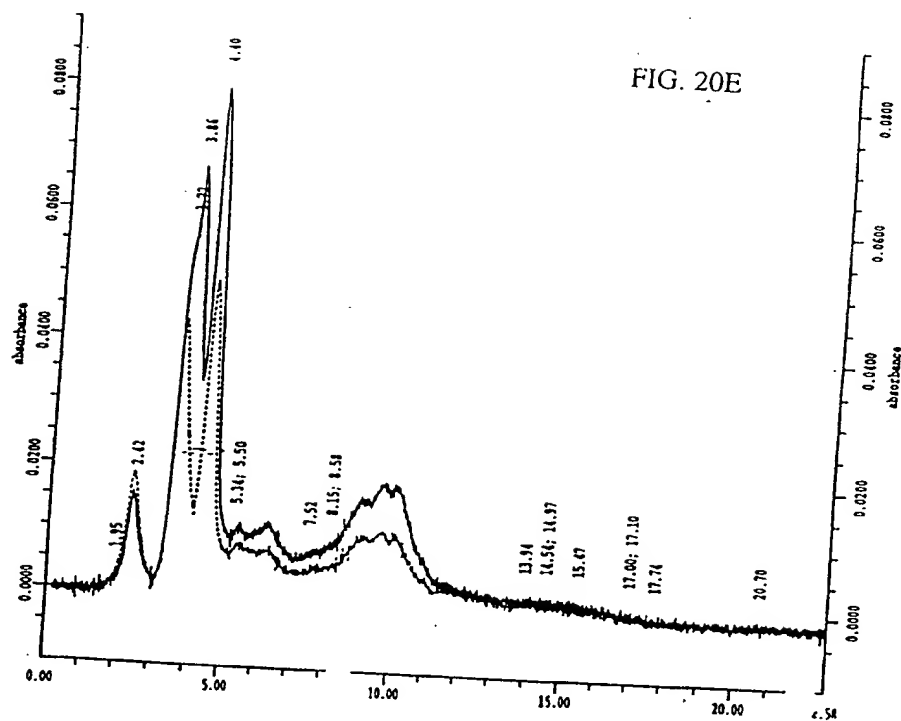
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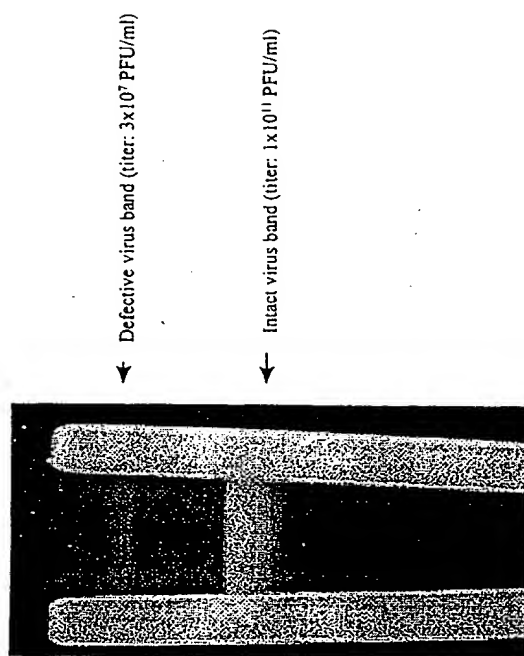
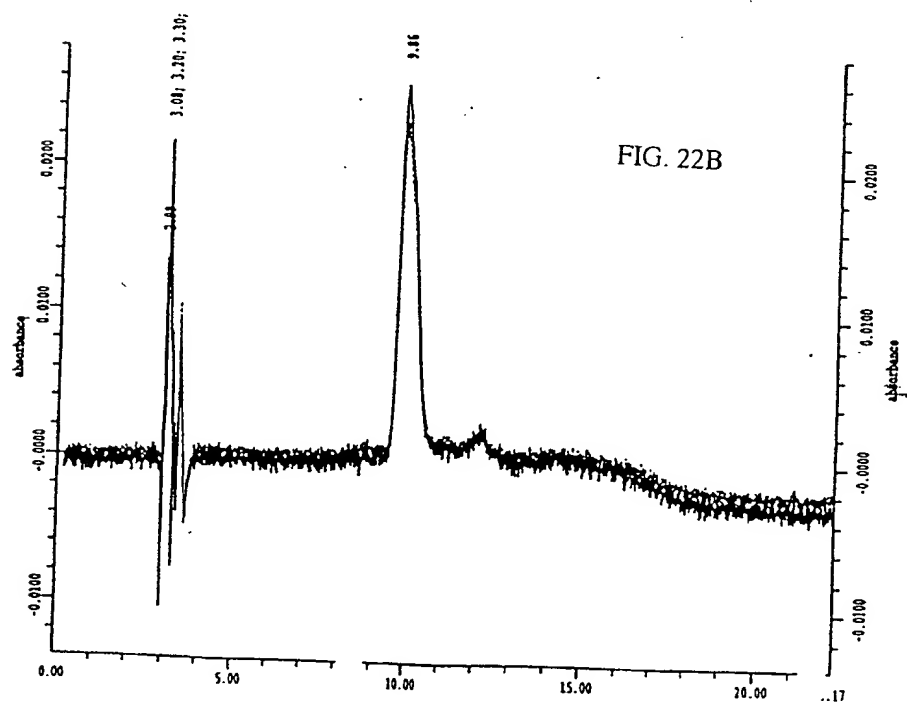
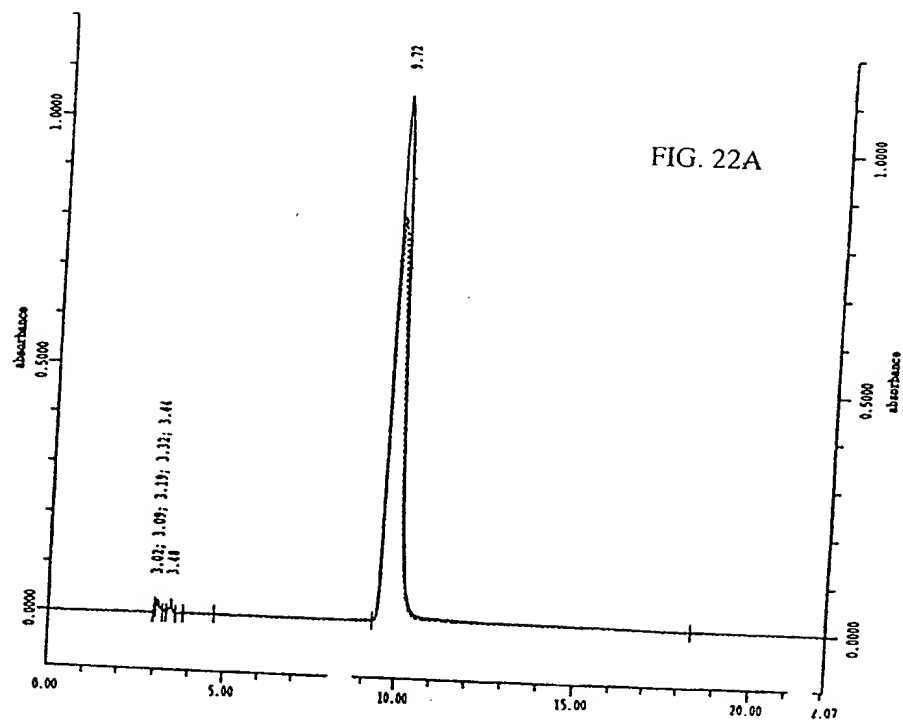


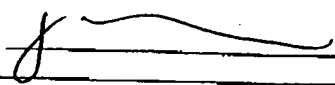
FIG. 21

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	Titer (PFU/ml)	Vol. (ml)	Yield (PFU)	Recovery (%)	
				Step	Acc.
Cube (low perfusion rate, keep glucose > 1g/L)					
↓ 1% Tween-20 in buffer A					
Harvest					
↓ Clarification and Filtration (0.22 μ m)					
Virus solution	2.6×10^9	1900	4.9×10^{12}		
↓ Conc./diaf. (10-fold conc., diaf. into 1M NaCl buffer A)					
Conc. sup	2.5×10^{10}	200	5×10^{12}	102%	
↓ Benzonase treatment (O/N, RT, 100u/ml)					
Treated sup					
↓ Dilute with water to conductivity = 22-25 mS/cm					
Diluted virus solution	7×10^9	700	4.9×10^{12}	98%	100%
↓					
Purified virus	1.5×10^{10}	240	3.6×10^{12}	73%	73%
↓ conc./diaf (5-fold conc.)					
Final purified product	7×10^{10}	50	3.5×10^{12}	97%	71%

FIG. 23

<p align="center">CERTIFICATE OF MAILING 37 C.F.R. §1.8</p> <p>I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450, on the date below:</p>	
<p>9/3/04 Date</p>	

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Shuyuan Zhang, Capucine Thwin, Zheng Wu,
Toohyon Cho, Shawn Gallagher

Group Art Unit: 1645

Examiner: Shanon A Foley

Serial No.: 09/203,078

Atty. Dkt. No.: INRP:081

Filed: December 1, 1998

For: METHOD FOR THE PRODUCTION AND
PURIFICATION OF ADENOVIRAL
VECTORS

SECOND DECLARATION OF SHUYUAN ZHANG UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

I, Shuyuan Zhang, do declare that:

1. I am a inventor of the claims in the above-referenced application.
2. I am also an inventor of U.S. Patent 6,194,191 ("the '191 patent"); which I understand to be the same disclosure as WO 98/22588 ("PCT application") (collectively "Zhang disclosures").
3. I understand claims 1-32, 38-49, and 51-62 in the above-referenced application have been rejected as anticipated or obvious in the Office Action dated June 3, 2004 ("Action")

based on the Zhang disclosures. Claims 1 and 47 in the rejected set of claims are independent claims, and the remaining claims are dependent from either claim 1 or claim 47.

4. I understand that the Action asserts that the Zhang disclosures anticipate "a process of preparing adenovirus by preparing a culture of producer cells essentially homogenous with respect to growth, infecting the producer cells with adenovirus between mid-log and stationary phase, and harvesting the adenovirus and placing the virus into a pharmaceutically acceptable composition." Action at page 9.
5. This process was invented by me and the other inventors common to the above-referenced patent application and the inventors listed on the cited Zhang disclosures.
6. Furthermore, in studies conducted at Introgen Therapeutics involving the growth of 293 cells in T-150 flasks, a cell doubling time of approximately 30 hours during exponential growth phase was observed. See pages 9 and 12 of Appendix A (Quality Assurance Report).
7. I hereby declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the referenced patent application or any patent issued thereon.

02 Sep 04

Date

23448840.1



Shuyuan Zhang, Ph.D

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,194,191
DATED : February 27, 2001
INVENTOR(S) : Zhang et al.

Page 1 of 2

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

On the title page, item [54], title, please insert --AN IMPROVED -- before "METHOD FOR THE PRODUCTION" therefor.

In claim 1, column 59, lines 34-37, please delete

- "b) providing nutrients to said host cells by perfusion or through a fed-batch process;
- c) infecting said host cells with an adenovirus;"

and insert

- b) infecting said host cells with an adenovirus;
- c) providing nutrients to said infected host cells by perfusion or through a fed-batch process; -- therefor.

In claim 30, column 60, lines 51-54, please delete

- "b) providing nutrients to said cells by perfusion or a fed-batch process at a rate to provide a glucose concentration of less than 2.0 g/L;
- c) infecting said host cells with an adenovirus, and"

and insert

- b) infecting said host cells with an adenovirus; and
- c) providing nutrients to said infected host cells by perfusion or a fed-batch process at a rate to provide a glucose concentration of less than 2.0 g/L; -- therefor.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,194,191
DATED : February 27, 2001
INVENTOR(S) : Zhang et al.

Page 2 of 2

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In claim 56, column 61, line 56, please delete "claim 55" and insert -- claim 53 -- therefor.

In claim 61, column 62, lines 4-6, please delete

"b) providing nutrients to said host cells by perfusion or through a fed-batch process;
c) infecting said host cells with an adenovirus;"

and insert

--b) infecting said host cells with an adenovirus;
c) providing nutrients to said infected host cells by perfusion or through a fed-batch process; -- therefor.

Signed and Sealed this

Fifth Day of June, 2001

Nicholas P. Godici

NICHOLAS P. GODICI

Attest:

Attesting Officer

Acting Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,194,191 B1
DATED : February 27, 2001
INVENTOR(S) : Zhang et al.

Page 1 of 2

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,

Item [54], title, please insert -- **AN IMPROVED** -- before "METHOD FOR THE PRODUCTION" therefor,

Column 59, claim 1,

Lines 34-37, please delete

- "b) providing nutrients to said host cells by perfusion or through a fed-batch process;
- c) infecting said host cells with an adenovirus;"

and insert

- b) infecting said host cells with an adenovirus;
- c) providing nutrients to said infected host cells by perfusion or through a fed-batch process; -- therefor.

Column 60, claim 30,

Lines 51-54, please delete

- "b) providing nutrients to said cells by perfusion or a fed-batch process at a rate to provide a glucose concentration of less than 2.0 g/L;
- c) infecting said host cells with an adenovirus, and"

and insert

- b) infecting said host cells with an adenovirus; and
- c) providing nutrients to said infected host cells by perfusion or a fed-batch process at a rate to provide a glucose concentration of less than 2.0 g/L; -- therefor.

Column 61, claim 56,

Line 56, please delete "claim 55" and insert -- claim 53 -- therefor.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,194,191 B1
DATED : February 27, 2001
INVENTOR(S) : Zhang et al.

Page 2 of 2

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 62, claim 61,
Lines 4-6, please delete

- "b) providing nutrients to said host cells by perfusion or through a fed-batch process;
- c) infecting said host cells with an adenovirus;"

and insert

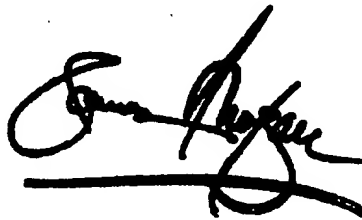
- b) infecting said host cells with an adenovirus;
- c) providing nutrients to said infected host cells by perfusion or though a fed-batch process; -- therefor.

This certificate supersedes Certificate of Correction issued June 5, 2001.

Signed and Sealed this

Twenty-ninth Day of January, 2002

Attest:



Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,194,191 B1
DATED : February 27, 2001
INVENTOR(S) : Zhang et al.

Page 1 of 2

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page,

Item [54], title, please insert -- **AN IMPROVED** -- before **"METHOD FOR THE PRODUCTION"** therefor,

Column 59, claim 1,

Lines 34-37, please delete

- "b) providing nutrients to said host cells by perfusion or through a fed-batch process;
- c) infecting said host cells with an adenovirus;"

and insert

- b) infecting said host cells with an adenovirus;
- c) providing nutrients to said infected host cells by perfusion or through a fed-batch process; -- therefor.

Column 60, claim 30,

Lines 51-54, please delete

- "b) providing nutrients to said cells by perfusion or a fed-batch process at a rate to provide a glucose concentration of less than 2.0 g/L;
- c) infecting said host cells with an adenovirus, and"

and insert

- b) infecting said host cells with an adenovirus; and
- c) providing nutrients to said infected host cells by perfusion or a fed-batch process at a rate to provide a glucose concentration of less than 2.0 g/L; -- therefor.

Column 61, claim 56,

Line 56, please delete "claim 55" and insert -- claim 53 -- therefor.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,194,191 B1
DATED : February 27, 2001
INVENTOR(S) : Zhang et al.

Page 2 of 2

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 62, claim 61,
Lines 4-6, please delete

- "b) providing nutrients to said host cells by perfusion or through a fed-batch process;
- c) infecting said host cells with an adenovirus;"

and insert

- b) infecting said host cells with an adenovirus;
- c) providing nutrients to said infected host cells by perfusion or through a fed-batch process; -- therefor.

This certificate supersedes Certificate of Correction issued January 29, 2002.

Signed and Sealed this

Seventeenth Day of December, 2002

A handwritten signature in black ink, appearing to read "James E. Rogan", written over a horizontal line.

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,194,191 B1
DATED : February 27, 2001
INVENTOR(S) : Zhang et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title page.

Item [54], title, please insert -- AN IMPROVED -- before "METHOD FOR THE PRODUCTION" therefor.

Column 61.

Line 56, please delete "claim 55" and insert -- claim 53 -- therefor.

This certificate supersedes Certificate of Correction issued December 17, 2002, and all previous Certificates of Corrections that issued June 5, 2001 and January 29, 2002.

Signed and Sealed this

Sixth Day of May, 2003

A handwritten signature in black ink, appearing to read "James E. Rogan", with a horizontal line drawn underneath it.

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

CHAPTER 11

Manipulation of Adenovirus Vectors

Frank L. Graham and Ludvik Prevec

1. Introduction

Adenoviruses are intermediate-sized DNA viruses with genomes, consisting of linear double-stranded DNA molecules of approx 36,000 bp. The virion is an icosahedron about 70 nm in diameter, consisting exclusively of protein and DNA. Adenoviruses have been isolated from a large number of different species (mammalian and fowl) and over 100 different serotypes have been reported, some 43 of them human. The human adenoviruses, particularly types 2, 5, and 12, have been the most extensively characterized, and these viruses have served as valuable tools in the study of the molecular biology of DNA replication, transcription, RNA processing, and protein synthesis in mammalian cells. *See ref. 1 for a general review.*

For a number of reasons, adenoviruses are attracting increasing attention as potential mammalian cell expression vectors and recombinant vaccines (for an excellent recent review of adenovirus vectors, *see ref. 2*). Not only is the viral particle relatively stable, but also, in the case of serotypes commonly used as vectors to date, the viral genome does not undergo rearrangement at a high rate, and insertions of foreign genes are generally maintained without change through successive rounds of viral replication. The adenovirus genome is also relatively easy to manipulate by recombinant DNA techniques, and the virus replicates efficiently in permissive cells. Infected cells

produce 1000–10,000 plaque-forming units (PFU), and the virus remains concentrated within the cell long after yields have reached maximum levels, making collection and concentration of virus extremely easy. Since a large fraction of the infected cell protein and DNA is viral at late stages of infection, adenoviruses are very attractive as vectors for high-level expression of proteins in mammalian cells. Furthermore, because adenoviruses can transform a variety of different cell types, resulting in the integration of viral DNA into the host-cell chromosome, adenoviruses carrying foreign DNA may serve as efficient gene transfer vectors in mammalian cells. Finally, the availability of over 40 different human serotypes, and of many different viruses isolated from other animals, affords considerable versatility in the selection of appropriate viruses for specific purposes.

This chapter outlines the methods for growing, titrating, and purifying adenoviruses; for extracting viral DNA from purified virions, and from infected cells; for rescuing inserts of foreign DNA into the viral genome; and for assessing the expression of inserted genes in adenovirus vectors. The details derive from our work with human adenovirus type 5 (Ad5), but the principles should apply to manipulation of other adenovirus serotypes.

Figure 1 shows a simplified map of the Ad5 genome, with a few key landmarks (for more details, *see ref. 1*). The replication cycle of the virus can be divided into two phases: early, corresponding to events occurring before the onset of viral DNA replication; and late, corresponding to the period after initiation of DNA replication. During the early phase, four noncontiguous regions of the genome are expressed: early region 1 (E1), which comprises E1a and E1b; E2; E3; and E4. After the onset of DNA replication, the major late promoter (MLP) located at 16 map units (m μ) drives much of the viral transcription. Transcription originating from the MLP terminates near the right end of the genome, and the late transcripts are processed into a complex array of different mRNAs (not shown in Fig. 1) that encode most of the structural virion proteins.

Some of the restriction enzyme sites most frequently used in the manipulation of Ad5 DNA are shown in Fig. 1. *Cla*I cuts only once near the left end (2.5 m μ) in wild-type Ad5 DNA; *Bam* HI cleaves wild-type DNA once at 59.5 m μ and *Eco*RI cuts twice, at 76.0 and 83.5 m μ . *Xba*I makes four cuts in wild-type DNA, but a useful mutant, *dB09* (3), has retained only the *Xba*I site at 3.7 m μ in E1—*dB09* DNA and the enzymes *Cla*I and *Xba*I are widely used in the construction of Ad5 mutants and vectors containing altered E1 sequences.

There are at least three regions of the viral genome that can accept insertions or substitutions of DNA to generate a helper independent virus. These are in E1, in E3, and in a short region between E4 and the end of the genome

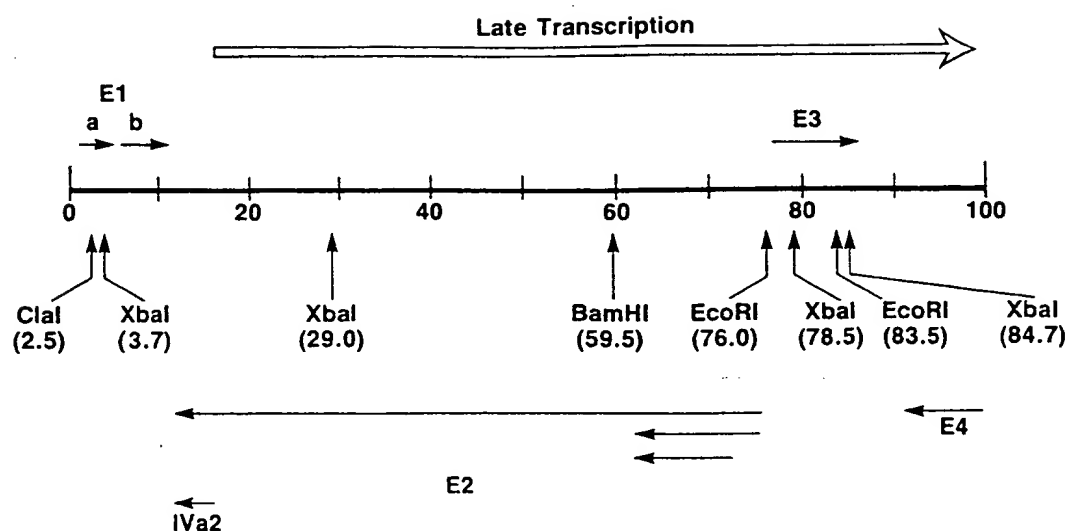


Fig. 1. Structure of the Ad5 genome. Locations of a few restriction enzyme sites are indicated in map units (mμ) (100 mμ = 100% = 36 kb). Arrows indicate transcriptional units.

(see ref. 2 and studies cited therein). Our work has involved insertions or substitutions, principally in E3 and to some extent in E1, and the methods outlined in the following sections deal specifically with construction of vectors having inserts of foreign genes in either of those two regions. However, with minor modifications, the techniques we describe should be applicable to insertion of DNA into any nonessential region of the genome.

E1 is not required for viral replication in human 293 cells (a line that is transformed by Ad5 DNA and contains and expresses the left end of the genome) (4), and E3 is nonessential for replication of adenovirus in cultured human cells. The most DNA that can be packaged in virions is approx 105% of the wild-type genome (5), for a capacity of about 2 kb of extra DNA. To incorporate larger DNA segments, it is necessary to compensate by deleting appropriate amounts of viral DNA. One of the most useful deletions is made by collapsing the two naturally occurring XbaI sites within E3 (Fig. 1) to remove 1.9 kb of viral DNA (6,7). This results in vectors having a capacity for approx 4 kb of foreign DNA and having the ability to replicate in any of the cell lines commonly used for adenovirus propagation, such as HeLa or KB cells. Approximately 3 kb can be deleted from E1 to generate vectors restricted to growth in 293 cells and able to accept inserts of 5 kb. Combining E1 and E3 deletions in a single vector should result in a capacity of approx 7 kb. It is important to point out, however, that the E1 deletion must not extend into the E1 region containing the coding sequences for protein IX

(from 10 to 11 m μ), since protein IX is a virion structural component that is necessary for packaging full length viral genomes, and deleting this gene results in a net decrease in capacity (5). If the generation of helper/dependent vectors is a suitable option, then, except for the extreme terminal sequences (which must be retained to allow DNA replication) and sequences near the left end (which are needed in *cis* for packaging), theoretically, almost the entire genome (approx 35 kb) can be substituted with foreign DNA.

Most of the nondefective vectors constructed by us and others have the 1.9 kb deletion in the E3 region and use the XbaI site at this position as a cloning site. Inserts in E3 can have either the E3 parallel or the E3 anti-parallel orientation; this is an important distinction, because expression of E3 inserts in the parallel (left to right) orientation seems to be primarily a result of transcripts originating from somewhere to the left in the viral genome, either from the E3 promoter or from the MLP (cf. 8-10).

There are certain basic steps that are followed prior to rescuing genes into the viral genome. These involve relatively straightforward recombinant DNA manipulations in which the gene to be cloned in viral DNA is first inserted into a subsegment of the viral genome, itself cloned in a bacterial plasmid. The resulting chimeric construct is then cotransfected into mammalian cells, along with appropriately prepared viral DNA, by conventional DNA transfer techniques. Usually, rescue is achieved by *in vivo* recombination in the transfected cells, but prior *in vitro* ligation is also an option. Figure 2 illustrates the procedure for rescue of either E1 insertions (shown below the Ad5 map) or E3 insertions (above map) into the viral genome by cotransfection with appropriately restricted viral DNA (cleavage of viral DNA reduces the infectivity of the parental viral DNA and enhances the efficiency of isolation of recombinant vectors resulting from *in vivo* recombination).

For E1 inserts, the first requirement is a bacterial plasmid containing the left end of the genome and having an appropriate deleting E1 sequences and a restriction site into which to clone a foreign gene. In the example shown in the lower part of Fig. 2, the requisite plasmid is pXCX2 (11). This is a plasmid containing the left 16% of the Ad5 genome cloned in pBR322, minus a deletion in E1 from 1.3 to 9.3 m μ , and having a unique XbaI cloning site for insertion of foreign DNA. The resulting construct is cotransfected into 293 cells along with virion DNA, usually derived from d β 309 virus, that has been cleaved at the left end to eliminate or at least reduce infectivity of the parental viral DNA. *In vivo* recombination results in rescue of the cloned viral sequences into the left end of the genome. Rescue into E3 is a similar process. In this case, one starts with a plasmid such as pFGdX1 (7), which con-

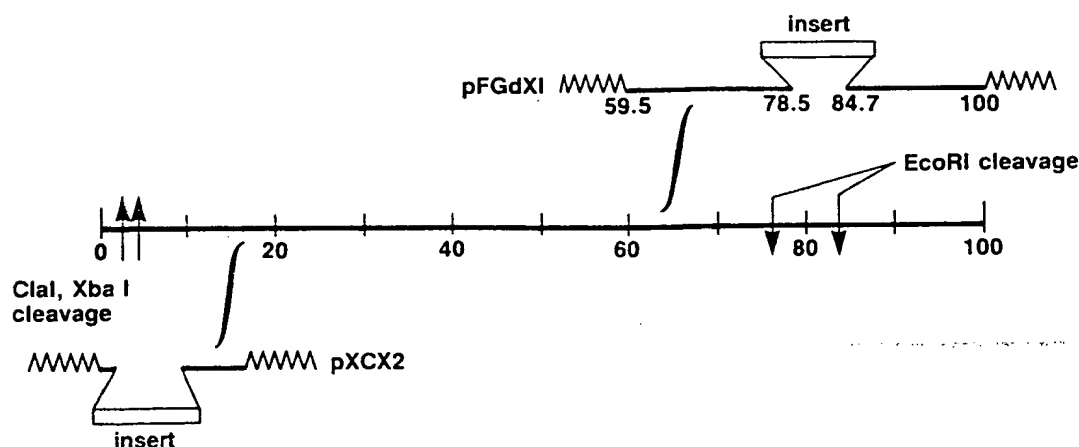


Fig. 2. Strategy for rescue of foreign DNA into the Ad5 genome. Rescue by in vivo recombination is shown schematically for inserts in region E1 (below genome map) or in region E3 (above map). Ad5 sequences are indicated by solid lines, insertions by open bars, and bacterial plasmid sequences, which are linked to viral DNA in plasmids pFGdX1 and pXCX2, by jagged lines. Relevant restriction enzyme digestion sites are indicated for rescue of E3 insertions (EcoRI digestion of wild-type virion DNA) or E1 insertions (ClaI and XbaI digestion of *dl309* DNA). See text for further details.

tains the right 40% of the genome from 59.5 to 100 mμ with a deletion in E3 and, again, a unique XbaI restriction enzyme site for cloning. Wild-type virion DNA is cut with EcoRI as shown and cotransfected with plasmid DNA, again resulting in recombination in vivo. Resulting plaques are isolated, expanded, and screened by restriction analysis to identify the desired recombinant. These methods are usually satisfactory, but there is often a background of infectious parental DNA, which can be a serious problem if the desired vector replicates significantly slower than wild-type virus.

To circumvent potential problems posed by infectious parental virion DNA, we have developed alternative methods that became feasible following the discovery that adenovirus DNA can circularize in infected cells (12), and that circular forms of the genome can be cloned as infectious plasmids (13,14). One such plasmid, pFG140, has been described previously (13) and consists of a circular *dl309* genome with an insert at 3.7 mμ of a small 2.2 kb DNA segment carrying ampicillin resistance and a bacterial origin of replication that permits propagation in *E. coli*. From pFG140, a series of simple recombinant DNA manipulations was used to substitute the 2.2-kb insert with a 4.4

kb DNA segment, resulting in a plasmid designated pJM17 (15). Because an insertion of 4.4 kb is too large to be packaged into infectious virions, co-transfection with left-end sequences containing substitutions or insertions that do not exceed the packaging constraints selects for recombination and rescue of the E1 inserts into infectious virus. Similar approaches have been used to construct another pFG140 derivative, pFG173 (F. L. Graham, unpublished), with a lethal deletion around the E3 region to select for recombination with viral DNA spanning that part of the genome. Neither of the co-transfected plasmids need be digested with restriction enzymes to obtain *in vivo* recombination. The advantages of this approach are that the background of infectious parental DNA is zero (when pFG173 is used) or low (using pJM17) and, once the appropriate plasmids have been made, purification and restriction of viral DNA can be avoided. We have used both conventional and these newer techniques to construct over 20 vectors expressing various gene products. The protocols described below are ones that have been used routinely in the course of that work.

2. Materials

2.1. Propagation, Titration, and Purification of Adenovirus

1. Cell lines and media: Wild-type and E3-Ad5 can be propagated in most standard human cell lines, such as HeLa or KB; E1-mutants are propagated in 293 monolayer cells (4) or in 293N3S suspension cultures (16). For all DNA transfections and most virus plaque assays, we recommend 293 cells. Any of the standard growth media, such as Minimal Eagle's Medium (MEM), α -MEM, or MEMF 11, supplemented with bovine serum (e.g., 10% fetal bovine serum or newborn calf serum), can be used for cell culture. We generally use MEMF 11 + either 2 or 5% heat-inactivated horse serum (HS) for propagation of virus.
2. Phosphate buffered saline (PBS²⁺) is prepared as follows:
 Solution A: 80 g NaCl, 2 g KCl, 11.5 g Na₂HPO₄, 2 g KH₂PO₄ /L of distilled H₂O;
 Solution B: 1 g CaCl₂·2H₂O/100 mL H₂O; and
 Solution C: 1 g MgCl₂·6H₂O/100 mL H₂O.
 Sterilize solutions A, B, and C separately by autoclaving. For 1L of PBS²⁺, mix 880 mL of sterile H₂O with 100 mL of solution A, and then add 10 mL each of solutions B and C. For 1L of PBS²⁻, mix 900 mL of H₂O with 100 mL of Solution A.
3. Overlay for plaque assays and transfections: Prepare 400 mL of 2 × MEMF

11 + 100 U/mL penicillin + 100 µg/mL streptomycin + 8 mL of 5% yeast extract. This can be stored for a few weeks at 4°C. To make 200 mL of complete overlay (20 60-mm dishes) prepare 100 mL of 2 × MEMF 11 + 10 mL inactivated HS, and autoclave 100 mL H₂O + 1 g agarose. Bring the agarose and F11 to 44°C before mixing and use within about 1 h.

4. Crystal violet for fixing and staining cell monolayers: Dissolve 2 g crystal violet in 20 mL methanol, and add 144 mL PBS²⁻ and 36 mL formaldehyde. Filter through Whatman no. 1 filter paper to remove any particulate matter prior to use.
5. Neutral red is purchased as a sterile stock solution of 0.33g/L neutral red (sodium salt).
6. 10% glycerol in PBS²⁻. Glycerol can be sterilized by autoclaving prior to mixing with PBS.
7. Tris-HCl buffer, 0.1 M and 0.01 M, pH 8.0.
8. Carnoy's fixative: methanol:glacial acetic acid, 3:1.
9. 1% sodium citrate: 1 g sodium citrate dihydrate dissolved in 100 mL H₂O.
10. Orcein staining solution: 2% orcein in 50% acetic acid. Filter through Whatman No. 1 paper.
11. Sodium deoxycholate: 5 g/100 mL H₂O.
12. Saturated CsCl: At room temperature, add sufficient CsCl to 0.01 M Tris-HCl, pH 8.0; 0.001 M EDTA to saturate the buffer. Store at 4°C, but bring to room temperature prior to use.

2.2. Adenovirus DNA Purification

1. 0.01 M Tris-HCl, pH 8.0.
2. Pronase stock solution: Dissolve pronase at 5 mg/mL in 0.01 M Tris-HCl, pH 7.5; preincubate at 56°C for 15 min, and then incubate at 37°C for 1 h. Aliquot into plastic tubes and store at -20°C. Pronase solution is thawed and diluted in buffer just prior to use for DNA extraction from purified virus (1 mg/mL pronase in 0.01 M Tris-HCl, pH 7.5; 0.01 M EDTA; 1% SDS) and from infected cell monolayers (0.5 mg/mL pronase in 0.01 M Tris-HCl, pH 7.5; 0.01 M EDTA; 0.5% SDS).
3. Phenol: Distilled or nucleic-acid grade phenol is melted and saturated with 0.01 M Tris-HCl, pH 8.0; 0.01 M EDTA. We generally add a few crystals of hydroxyquinoline, which not only inhibits oxidation but also gives a distinctive orange color to the phenol, thus facilitating discrimination between aqueous and phenol phases in DNA extraction procedures.
4. 30% sodium acetate trihydrate.
5. 0.1 × SSC is usually made from 20 × solutions by diluting in H₂O and

autoclaving to sterilize and inactivate nucleases ($20 \times$ SSC: 175 g NaCl and 88 g sodium citrate dihydrate/L).

2.3. DNA Transfection and Screening of Plaque Isolates

1. HEPES-buffered saline (HEBS): 5 g/L HEPES (*N*-2-Hydroxyethyl-piperazine-*N*-2-ethanesulfonic acid) 8 g/L NaCl, 0.37 g/L KCl, 0.125 g/L $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1 g/L glucose; final pH 7.1. HEBS is aliquoted into small glass bottles, sterilized by autoclaving, and stored at 4°C with caps tightly closed.
2. Carrier DNA: We use commercially available salmon-sperm DNA. This is dissolved in sterile $0.1 \times$ SSC at approx 1 mg/mL, the exact final concentration determined by OD at 260 nm. This stock solution is stored in small aliquots at -20°C and used at a final concentration of 10 $\mu\text{g/mL}$ HEBS.
3. 2.5M CaCl_2 : Prepared in distilled H_2O , sterilized by filtration, and stored in small plastic tubes or flasks at 4°C .
4. Restriction enzymes, apparatus for horizontal slab gel electrophoresis, and the like, for analyzing structure of recombinant viruses.

2.4. Detection of Expression

1. Cell-culture media: Complete MEMF 11 supplemented with 5% horse serum; medium lacking methionine (MEMmet⁻) is usually MEM119 with no methionine, supplemented with 2% dialyzed fetal bovine serum.
2. ^{35}S -methionine: Either L-methionine [^{35}S] with a specific activity >800 Ci/mmol or a mixture of predominantly L-methionine [^{35}S] and L-cysteine [^{35}S] can be used.
3. PBS⁻: Described in Section 2.1.2.
4. RIPA buffer: 0.05M Tris-HCl (pH 7.2), 0.15M NaCl, 0.1% (w/v) SDS, 1% (w/v) sodium deoxycholate, and 1% (v/v) Triton X-100.
5. Protein A Sepharose beads (Pharmacia Inc.) are prepared by swelling 500 mg of beads in 25 mL of RIPA buffer on a roller wheel at 4°C . The beads are washed three times by centrifugation at 1500 rpm for 5 min and resuspended in RIPA buffer. The final pellet is resuspended in 3.5 mL of RIPA buffer and stored at 4°C .
6. Loading buffer: 5% (w/v) SDS, 12.5% (v/v) β -mercaptoethanol, 25% (v/v) glycerol, and 0.1% (w/v) bromophenol blue.
7. Apparatus and materials for SDS polyacrylamide gel electrophoresis of proteins and subsequent detection of radioactive proteins on X-ray film.

3. Methods

3.1. Propagation, Titration, and Purification of Adenovirus

3.1.1. Plaque Assays

1. Set up appropriate cells (HeLa or 293) 1 d prior to use, arranging to have the cells just at confluency when used. The actual split ratio depends on the cell type, but a good rule of thumb is about 6–8 60-mm dishes from each 150-mm dish of 293 cells, and about 20–30 60-mm dishes from each 150-mm dish of HeLa cells.
2. The following day, prepare virus dilutions in PBS²⁺. Remove the medium and add 0.2 mL virus/dish. Adsorb the virus at room temperature for 30–60 min, occasionally tipping the dishes to spread the suspension over the cell monolayer. To each 60-mm dish, add 10 mL of overlay that has been prepared beforehand and has been equilibrated in a 44°C waterbath.
3. Incubate at 37°C in a CO₂ incubator. On 293 cell monolayers, plaques should be visible within 4–5 d and can be counted at 6–8 d. Plaques on 293 cells can be counted by eye, or the cells can be fixed with formaldehyde, the overlay can be flipped out, and the monolayer stained with crystal violet. Plaques on HeLa cells can be counted after staining with neutral red by overlaying on d 7 or 8 with 5 mL of overlay containing 33 µg/mL neutral red and counting plaques 2–3 days later.

3.1.2. Infection of Cells in Monolayer

1. After determining the number of cells on a dish that is about 80–90% confluent, remove the medium and add appropriately diluted virus in 1 mL PBS²⁺/150-mm dish, or 0.2 mL/60-mm dish at multiplicity of infection (moi) of 1–10 PFU/cell.
2. Adsorb for 30–60 min, tipping the dishes once or twice to spread the suspension evenly and refeed with MEMF 11 + 2% heat-inactivated horse serum. Incubate the dishes at the appropriate temperature and examine twice daily for signs of cytopathic effect. Virus replication and spread is more rapid in 293 cells, and cytopathic effect is more readily apparent than in infected HeLa or KB cells.
3. When the cytopathic effect is complete (as indicated by rounding up of the cells), harvest by scraping the cells off the plastic and centrifuging infected cells from the medium. For a relatively concentrated stock of virus, resuspend the cell pellet in 2 mL PBS²⁺ + 10% glycerol/dish. Cells

can be broken and virus released by sonication or by freezing and thawing. The medium will also contain substantial amounts of infectious virus at a lower titer than the concentrated cell suspension, and may be useful for some purposes for which a high titer is not needed. Sterile glycerol should be added (to 10%) and the virus stored at -20 to -70°C .

3.1.3. Infection of Cells in Suspension (KB or 293N3S)

1. Grow spinner culture cells to $2-4 \times 10^5$ cells/mL in Joklik's modified MEM + 10% inactivated horse serum. Centrifuge gently to pellet cells, saving 50% of the conditioned medium, and resuspend the cell pellet in 1/10 vol of medium.
2. Add virus at a moi of 10–20 PFU/cell and stir gently at 37°C for 1 h.
3. Bring to the original vol., using 50% fresh medium and 50% conditioned medium, and continue incubating at 37°C . Monitor infection on a twice-daily basis using inclusion body staining (*see* Section 3.1.4.).
4. When inclusion bodies are visible in 80–90% of infected cells (about 11/2–2 d), harvest by centrifugation and resuspend in 10 mL PBS^{2+} +10% glycerol/L infected cells for preparation of stocks of crude infectious virus, or resuspend in 0.1 M Tris-HCl, pH 8.0 (10–20 mL for 1–4 L), for preparation of virus to be used for CsCl banding and DNA extraction.

3.1.4. Inclusion Body Staining

1. Remove a 5-mL aliquot from the infected spinner culture. Centrifuge for 10 min at 1000 rpm and resuspend the cell pellet in 0.5 mL of 1% sodium citrate.
2. Incubate at room temperature for 10 min; then add 0.5 mL Carnoy's fixative and fix for 10 min at room temperature.
3. Add 1 mL of 1% sodium citrate, centrifuge, and resuspend the pellet in a few drops of 1% sodium citrate. Add a drop of fixed cells to a slide and dry for at least 1 h; then add Orcein and a cover-slip and examine in the microscope. Inclusion bodies appear as densely staining nuclear structures resulting from accumulation of large amounts of virus and viral products at late times in infection. A negative control should be included in initial tests.

3.1.5. Purification of Adenovirus: A Rapid, Simple Method

Concentrated crude virus stocks are prepared from infected KB cells or 293N3S cells by pelleting cells infected as described above and resuspending the cell pellet in 5–10 mL of 0.1 M Tris-HCl, pH 8.0/L of infected cell suspension. This is stored at -70°C until needed.

1. Thaw the frozen crude virus stock and add 1/10 vol 5% sodium deoxycholate. Mix well and incubate on ice for 30 min. This disrupts cells without disrupting virions, and results in a relatively clear, highly viscous suspension.
2. Shear the cellular DNA using a probe-type homogenizer. We have found this step to be necessary to avoid aggregation of virus and cellular material, presumably DNA, during the subsequent CsCl banding step. Viscosity should be reduced so that, when the suspension is pipetted dropwise, there is still some noticeable viscosity, but only slightly more than that of water.
3. Add 1.8 mL saturated CsCl (in 0.01 M Tris-HCl, pH 8.0; 0.001 M EDTA) for each 3.1 mL of virus suspension. Be sure that the saturated CsCl stock is at room temperature prior to use, since this affects the concentration.
4. Distribute virus into Beckman 50Ti quickseal (or similar) tubes and spin in a Beckman 50Ti angle rotor (or a vertical rotor) for 16–20 h at 4°C and 35,000 rpm.
5. Collect the viral bands and pool. (For tubes other than nitrocellulose, one can collect by puncturing the top of the tube with a hot needle, then puncturing the bottom, and controlling the flow of solution out the bottom with a finger over the top hole.)
6. Centrifuge pooled virus in a Beckman SW50.1 rotor at 35,000 rpm, 4°C, for 16–20 h.
7. Collect the virus band. This time, try to collect the virus in a small vol. Banded virus is relatively stable at 4°C in CsCl, though it may eventually fall apart.

3.2. Adenovirus DNA Purification

This is a simple, reliable method for extracting virion DNA from CsCl-purified adenovirus. An alternative, somewhat more involved method is also available, which avoids proteases and phenol, and produces virion DNA with an intact terminal protein (cf. 17,18). Such DNA-protein complexes are much more infectious than deproteinized virion DNA; however, we do not use this method routinely, since we usually find specific infectivities of pure virion DNA made by the technique outlined below adequate for our purposes.

1. Dialyze the banded virus in boiled dialysis tubing for about 2 h against two changes of approx 100 vol of 0.01 M Tris-HCl, pH 8.0 (keep cold). Often, this causes the virus to precipitate, but for DNA extraction, this is no problem.

2. Prepare the digestion buffer containing 1 mg/mL pronase in 0.01M Tris-HCl, pH 7.5; 0.01M EDTA; 1% SDS.
3. To a Petri dish, add 1 vol of digestion buffer from step 2, and then add the dialyzed virus (1 vol) dropwise, mixing by tipping the dish. The order is important: Always add virus to pronase-SDS digestion buffer, rather than the reverse. Incubate for 2 h at 37°C.
4. Extract once with phenol that has been saturated with 0.01M Tris-HCl, pH 8.0; 0.01M EDTA.
5. Add 1/10 vol of 30% sodium acetate, ethanol-precipitate by adding 2 vol of 96% ethanol, and wash extensively with 96% ethanol. Dry for about 1 h at 37°C and redissolve DNA in $0.1 \times$ SSC. Expect to obtain about 0.5–1 mg DNA from each liter of original infected cells.
6. Aliquot DNA into several tubes and store at -20°C . Repeated freezing and thawing can degrade viral DNA; otherwise, it should be stable indefinitely.

3.3. Isolation of Expression Vectors by Cotransfection and In Vivo Recombination

This section outlines methods for cotransfecting 293 cells with plasmid and virion DNA, or with two plasmids, for rescue of insertions into the viral genome by recombination in vivo. See Fig. 2 for a general outline of the steps involved. If virion DNA is being used, it should be cut with appropriate restriction enzymes, as illustrated in Fig. 2, to reduce the infectivity of parental DNA and enhance the efficiency of recovery of recombinants. If two plasmids containing overlapping viral sequences are used, they should be engineered such that neither, on its own, is capable of generating infectious virus.

3.3.1. DNA Transfection

This is a more-or-less standard protocol for assaying infectivity of adenovirus DNA or for rescue of cloned viral sequences into full length viral genomes by cotransfection. Whether insertions are targeted to E1 or to other regions of the genome, the cell line of choice is always 293, because these cells are good recipients of DNA in DNA-mediated gene-transfer procedures, and generate adenovirus plaques rapidly and efficiently.

We routinely use the procedure outlined below to obtain infectious virus from adenovirus DNA or from plasmids containing the Ad genome. It is essentially unchanged from the original technique for assaying infectious Ad5 DNA (19). We have not investigated other methods; the reader is encouraged to evaluate some of the other transfection techniques described in this vol.

The 293 cells should be at low passage (<p50), should be set up the previous day in 60-mm dishes, and should be about 70–80% confluent at the time of use. Do not use growth media with high concentrations of phosphate, such as Joklik's modified MEM, since this interferes with transfections by the calcium phosphate technique.

1. Prepare 1× HEPES-buffered saline (HEBS) + 10 µg/mL salmon-sperm DNA (or other suitable carrier DNA), and mix well by vortexing for 1 min.
2. Aliquot HEBS + carrier DNA into sterile clear plastic tubes (e.g., at 0.5 mL/60-mm dish, four dishes will require 2 mL of HEBS).
3. Add the experimental DNA and mix well. We typically use 5–10 µg plasmid DNA containing the foreign DNA insert, and 2–5 µg restricted virion DNA or 5–10 µg of the second plasmid for each transfected dish.
4. Slowly add 2.5 M CaCl₂ (50 µL/mL) for a final concentration of 125 mM.
5. Mix well and incubate at room temperature for 15–30 min. (A fine precipitate should form within a few min.) Add the suspension to the cells without removing the growth medium (0.5 mL/5 mL medium) and incubate at 37°C in a CO₂ incubator for 4.5 h. Remove the medium and overlay with MEMF11 + 5% horse serum + 0.5% agarose, prepared as described above, or refeed with MEMF11 + 5% horse serum and incubate at 37°C. Plaques or cytopathic effect should appear after about 5–7 d.

3.3.2. Screening Adenovirus Plaque Isolates

Most screens will utilize 293 cells for expanding plaque isolates because the virus spreads rapidly, and because 293 cells are used for isolating many mutants (e.g., E1 mutants). The protocol below is designed for 293 cells.

1. Set up 60-mm dishes of 293 cells as for plaque assays, i.e., about 80–90% confluent. The denser and older the cell monolayer, the longer it takes for virus cytopathic effect to reach completion. Use dishes the next day.
2. Pick well-isolated plaques from transfected cultures (*see* Section 3.3.1.) by punching out agar plugs using a sterile Pasteur pipet, and transfer mashed agar to 1 mL of PBS²⁺ + 10% glycerol. This can be stored at –70°C until results of the analysis are available.
3. Remove the medium from 293 cell dishes and add 0.2 mL of virus. Distribute over the cell monolayer and adsorb at room temperature for 30 min. Add 5 mL MEMF 11 + 5% HS and incubate at 37°C.
4. Depending on the size of the original plaque and the growth properties of the virus mutant, a cytopathic effect should become visible within 1–2 d. Do not attempt to harvest before the cytopathic effect is absolutely complete, i.e., essentially all cells rounded and many floating (usually 3–4 d).

5. Process the dishes with complete cytopathic effect as follows: Leave dishes undisturbed in the tissue-culture hood for 20–30 min. Gently remove medium with a pipet and save about 4 mL in a sterile glass vial containing 0.5 mL sterile glycerol for storage at -70°C . Remove the residual medium by suction. If all this is done carefully, the majority of loose cells will be left behind in the dish.
6. Add 0.5 mL pronase (0.5 mg/mL pronase + 0.5% SDS prepared as in Section 2.2.) and digest at 37°C for 3–4 h.
7. Transfer the viscous lysate to a 1.5-mL Eppendorf tube (do this by leaving the dishes at an angle so the lysate drains to one edge of dish, and then collecting with a Pasteur pipet) and extract once with 0.5 mL of phenol. Collect the aqueous phase and transfer to a fresh tube.
8. Add 50 μL of 30% sodium acetate and precipitate with 1 mL of 96% ethanol; vortex or mix by tipping the tube. You should get an easily visible fibrous precipitate. Spin and wash twice with 1.5 mL of 96% ethanol to remove phenol.
9. Dry the pellet of crude DNA completely, redissolve in 50 μL of $0.1 \times \text{SSC}$ (complete solubilization may take several hours) and digest 5 μL with HindIII (this is the best all-purpose diagnostic enzyme for preliminary analysis of Ad5 DNA) for 3–4 h or, preferably, overnight.
10. Run on a 1% agarose gel with appropriate markers (a HindIII digest of wild-type Ad5 DNA being the best marker) and identify the candidate recombinants. If the cytopathic effect was complete, this procedure should result in a relatively pure preparation of viral DNA, with cellular DNA running as a background smear. There should be very little RNA. Note that in HindIII digests of human DNA, there will be a band of cellular repetitive DNA at around 1.8 kb, not to be confused with viral DNA. Candidates with predicted HindIII restriction patterns should be analyzed with additional enzymes, plaque-purified (using virus from the original agar plug), and reanalyzed.

3.4. Detection of the Expression of Inserts in Adenovirus Vectors

3.4.1. Expression in Virus-Infected Cell Cultures

The most suitable procedure for the detection of a product expressed by adenovirus vectors will, of course, depend on the properties of the protein produced and on the availability and quality of reagents necessary to detect it. For example, products with enzymatic or biological activity may be detected in vector-infected cell extracts or culture fluids using the appropriate

assays, whereas foreign-virus glycoproteins with fusion or hemeadsorption activity may be detected on the surface of vector-infected cells using these particular properties. The most generally applicable procedures depend on the ability to detect the expressed protein with conventional antigen-antibody detection procedures, such as ELISA, immunoprecipitation, or Western-blot analysis. The following protocol for immunoprecipitation is one used in our laboratory and, though satisfactory for most purposes, it is far from perfect in view of variable nonspecific precipitation of adenovirus proteins, which may obscure some of expressed products. This procedure of course depends on having a suitable precipitating antibody against the relevant protein, with no contaminating antiadenovirus activity.

1. Infect nearly confluent Hela cell monolayers in 60-mm Petri dishes with the adenovirus vector or with control adenovirus at a moi of 20 PFU/cell, as described in Section 3.1.2. Incubate in MEMF11 + 5% horse serum at 37°C.
2. At 12, 24, and 36 h postinfection, carefully remove medium from the infected cells and wash the cells once with prewarmed MEMmet- medium. Add 1 mL of MEMmet- medium containing 50 μ Ci of 35 S-methionine. Incubate for 2 h at 37°C in CO₂ incubator.
3. Remove the radioactive medium. Rinse the cell monolayer with PBS²⁻, and scrape the cells into PBS²⁻, using a rubber policeman. Pellet the cells by centrifugation for 15 min at 1500 rpm, remove the supernatant, and redissolve the cell pellet in 1 mL of RIPA buffer. Reduce the viscosity of the solution by passing the material through a syringe needle or by vigorous vortexing followed by sonication in an immersion bath sonifier. The former procedure is best initiated by drawing the solution up into the syringe barrel prior to attaching the needle, and then putting on the needle and expelling the material carefully through the needle into a tube.
4. Once the viscosity has been reduced to a point at which the solution flows reasonably freely, centrifuge in a microfuge for 15–30 min and collect the supernatant. (During the first few experiments, you may wish to save the pelleted material until you have determined that the protein of interest is not principally in this fraction; however, for most soluble and plasma-membrane-associated proteins, this will not be necessary.)
5. To 500 μ L of supernatant add an appropriate vol of antiserum (we generally use 5–10 μ L of serum). Then add 100 μ L of the suspension of

- protein A-Sepharose beads in RIPA buffer. Incubate the final suspension overnight at 4°C in a roller wheel or other suitable mixing device.
6. Pellet the beads by centrifugation for 5–10 min at 1500 rpm and wash the beads by repeated resuspension and pelleting (at least six times). The final pellet is resuspended in 50–100 µL of loading buffer.
 7. After 3 min in a boiling water bath, the sample is cooled and centrifuged to pellet the beads. The supernatant is then removed and analyzed by SDS polyacrylamide gel electrophoresis with appropriate controls and size markers, and the radioactivity is detected by autoradiography.

3.4.2. Expression In Vivo Detected by Antibody Production in Infected Animals

If the inserted protein may induce antibodies that can be readily and selectively detected (for example, if the insert encodes a virus glycoprotein that can induce virus neutralizing antibodies), then an indirect assay for expression of the insert in mice infected with the vector may be useful. We have found, in some cases in which reagents for immunoprecipitation were not available or not suitable, that the following method provided a sensitive test of antigen expression by the adenovirus vector, though this method is not necessarily suited to providing a quantitative measure of levels of expression. *Anyone intending to use this procedure should read carefully the section below relating to biohazards and should consult the relevant governing authorities before proceeding with these experiments.*

1. In general, crude lysates of cells infected with the Ad5 vector, prepared and titered as described above, will be suitable for this procedure, though in some instances, CsCl-purified virus may be required. In this latter case, care must be taken to dialyze extensively against PBS²⁺ + 10% glycerol to remove CsCl from the preparation before it is used. The virus should be titered by plaque assay after dialysis.
2. The vector (crude or purified) in 0.1-mL vol containing 10⁸ PFU is injected by the intraperitoneal route into recipient mice. We have routinely used 6- to 8-wk old Balb/C mice, but have no reason to believe that any other strain would not work as well.
3. The mice are bled by tail vein at 2 and 4 wk following immunization and the serum either titered for virus-neutralizing activity or used in an immunoblot or ELISA assay against purified antigen to detect specific antibodies. If a source of labeled antigen not contaminated with adenovirus antigens is available, then immunoprecipitation, PAGE, and autoradiography also can be used to detect the presence of specific antibody.

4. Notes

4.1. Biosafety Considerations in the Production and Use of Infectious Recombinant Vectors

Adenovirus vectors with foreign gene inserts in the E3 region are either directly or potentially infectious in humans or other permissive species. All experimentation with recombinant virus vectors should be carried out in accordance with regulations governing the use of these agents, and with the permission of relevant authorities. No known toxic (or potentially toxic) gene product should be expressed from adenovirus vectors.

Although Ad5 is a ubiquitous human virus infecting many individuals very early in life, there is nonetheless a significant fraction of individuals who do not carry antibodies to this virus. Individuals in this group would be particularly susceptible to infection with Ad5 vectors, and would be subject to seroconversion against any inserted foreign-gene products. This is to be avoided in most cases, especially if the development of antibody may confuse diagnosis or epidemiology of a particular disease. The same consideration should be given to possible animal infection with the vector.

4.2. Potential Pitfalls in the Isolation of Expression Vectors

1. The most common problems likely to be met in the construction of adenovirus expression vectors are, in order of likelihood: a. failure to obtain any plaques following cotransfection; b. failure to obtain recombinants among a large number of plaques screened; and c. failure of the insert to express following rescue.

Inability to obtain plaques can be the result of a number of factors, the most common and easiest to identify being poor transfection efficiencies. A positive control, such as uncut virion DNA, should generate 10–100 plaques/ μ g if the Ad DNA has been deproteinized, and 1–2 orders of magnitude more plaques if the terminal protein has been left intact. If the efficiency of plaque production with control DNA is poor, the most likely cause is the condition of the cell monolayer. Assuming 293 cells are being used, it is important that they be at an early passage, not be growing too rapidly, and be slightly less than confluent at the time of use. Suitable cell monolayers should yield visible plaques within 4–5 d following infection with virus or transfection with virion DNA. Other possible explanations for poor transfection efficiencies are incorrect HEBS composition (the pH, in particular, is critical and the

phosphate concentration is important as well), and poor-quality DNA, e.g., degraded or sheared viral DNA. If transfection efficiencies are satisfactory with control DNA, but no plaques are obtained with cotransfected cultures, check that the plasmid DNA is "clean." We generally use CsCl-banded plasmid DNA extracted from bacteria treated with chloramphenicol to amplify the plasmid copy number (*see* Chapter 1). Sometimes, linearizing the transfecting plasmid will enhance the recombination efficiencies. PFGdX1, for example, can be cut with BamHI if the insert is lacking BamHI sites. In a "worst-case scenario," transfections seem to be working, but only wild-type parental viruses are found by screening as many as 50–100 candidate plaque isolates obtained from cotransfections of restricted virion DNA and plasmid DNA. Assuming the virion DNA has been completely digested to reduce infectivity, then the unfortunate possibility exists that the insertion being rescued is potentially toxic for the cells or virus. This does not happen often, but it does happen. There is little that can be done if this is the case, but at least it may be possible to determine whether toxicity is the problem by introducing an inactivating mutation into the coding sequences of the insert (e.g., a chain terminator near the start for translation). If the construct now becomes rescuable, then toxicity is in all likelihood preventing replication of the vector carrying the original gene.

2. Our experience has been that vectors containing E3 inserts of foreign genes in the E3 parallel (left to right) orientation almost invariably express the inserted gene as protein, though the levels of expression can vary greatly from one insert to another. Whether the inserted gene has its own promoter or not, expression seems generally to be the result of transcription driven by upstream adenovirus promoters, either the E3 promoter or the major late promoter. In most of the vectors we have made and characterized, the gene to be expressed has been flanked by an upstream SV40 early promoter element and downstream poly A⁺ addition sequences, but we and others (10) have found that promoterless inserts in the E3 region also express protein in some vectors. Currently, obtaining expression of inserts in E3 is largely an empirical process and it is not yet possible to predict, *a priori*, the expression levels for any given insertion in E3. When inserts are located in E1, the use of a strong promoter upstream of the coding sequences is more-or-less essential, and a number of studies have been done with various promoters driving various genes inserted into the E1 region. The reader is referred to ref. 2 for further details and additional references.

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References

1. Ginsberg, H. S. (1984) *The Adenoviruses* (Plenum, New York).
2. Berkner, K. L. (1988) Development of adenovirus vectors for expression of heterologous genes. *BioTechniques* 6, 616-629.
3. Jones, N. and Shenk, T. (1979) Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* 16, 683-689.
4. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36, 59-72.
5. Ghosh-Choudhury, G., Haj-Ahmad, Y., and Graham, F. L. (1987) Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes. *EMBO J.* 6, 1733-1739.
6. Berkner, K. L. and Sharp, P. A. (1983) Generation of adenovirus by transfection of plasmids. *Nucleic Acids Res.* 11, 6003-6020.
7. Haj-Ahmad, Y. and Graham, F. L. (1986) Development of a helper independent human adenovirus vector and its use in the transfer of the Herpes Simplex Virus thymidine kinase gene. *J. Virol.* 57, 267-274.
8. Johnson, D. C., Ghosh-Choudhury, G., Smiley, J. R., Fallis, L., and Graham, F. L. (1988) Abundant expression of Herpes Simplex Virus glycoprotein gB using an adenovirus vector. *Virology* 164, 1-14.
9. Schneider, M., Graham, F. L., and Prevec, L. (1989) Expression of the glycoprotein of VSV by infectious adenovirus vectors. *J. Gen. Virol.* 70, 417-427.
10. Davis, A. R., Kostek, B., Mason, B. B., Hsiao, C. L., Morin, J., Dheer, S. K., and Hung, P. P. (1985) Expression of hepatitis B virus surface antigen with a recombinant adenovirus. *Proc. Natl. Acad. Sci. USA* 82, 7560-7564.
11. Spessot, R., Inchley, K., Hupel, T. M., and Bacchetti, S. (1989) Cloning of the herpes simplex virus ICP4 gene in an adenovirus vector: Effects on adenovirus gene expression and replication. *Virology* 168, 378-387.
12. Ruben, M., Bacchetti, S., and Graham, F. L. (1983) Covalently closed circles of human adenovirus type 5 DNA. *Nature* 301, 172-174.
13. Graham, F. L. (1984) Covalently closed circles of human adenovirus DNA are infectious. *EMBO J.* 3, 2917-2922.
14. Ghosh-Choudhury, G., Haj-Ahmad, Y., Brinkley, P., Rudy, J., and Graham, F. L. (1986) Human adenovirus cloning vectors based on infectious bacterial plasmids. *Gene* 50, 161-171.
15. McGrory, J., Bautista, D., and Graham, F. L. (1988) A simple technique for the rescue of early region I mutations into infectious human adenovirus type 5. *Virology* 163, 614-617.

16. Graham, F. L. (1987) Growth of 293 cells in suspension culture. *J. Gen. Virol.* 68, 937-940.
17. Sharp, P. A., Moore, C., and Haverty, J. L. (1976) The infectivity of adenovirus 5 DNA-protein complex. *Virology* 75, 442-456.
18. Chinnadurai, G., Chinnadurai, S., and Green, M. (1978) Enhanced infectivity of adenovirus type 2 DNA and a DNA-protein complex. *J. Virol.* 26, 195-199.
19. Graham, F. L. and van der Eb, A. J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52, 456-467.

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Scale-up of the adenovirus expression system for the production of recombinant protein in human 293S cells

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Key words: Adenovirus, human 293S cells, recombinant protein, scale-up, metabolism

Abstract

Human 293S cells, a cell line adapted to suspension culture, were grown to 5×10^6 cells/mL in batch with calcium-free DMEM. These cells, infected with new constructions of adenovirus vectors, yielded as much as 10 to 20% recombinant protein with respect to the total cellular protein content. Until recently, high specific productivity of recombinant protein was limited to low cell density infected cultures of no more than 5×10^5 cells/mL. In this paper, we show with a model protein, Protein Tyrosine Phosphatase 1C, how high product yield can be maintained at high cell densities of 2×10^6 cells/mL by a medium replacement strategy. This allows the production of as much as 90 mg/L of active recombinant protein per culture volume. Analysis of key limiting/inhibiting medium components showed that glucose addition along with pH control can yield the same productivity as a medium replacement strategy at high cell density in calcium-free DMEM. Finally, the above results were reproduced in 3L bioreactor suspension culture thereby establishing the scalability of this expression system. The process we developed is used routinely with the same success for the production of various recombinant proteins and viruses.

Abbreviations: CFDMEM – calcium-free DMEM; CS – bovine calf serum; hpi – hours post-infection; J+ – enriched Joklik medium; MLP – major late promoter; MOI – multiplicity of infection (# of infectious viral particle/cell); q – specific consumption rate (mole/cell.h); pfu – plaque forming unit (# of infectious viral particle); Y – yield ($\mu\text{g}/\text{E6 cells or mole/cell}$)

Introduction

The technological potential of adenovirus vectors (AV) in various applications such as 1) recombinant protein production, 2) live viral sub-unit vaccines production and 3) gene transfer for establishing stable cell lines or for gene therapy (reviewed in Berkner, 1988, 1992; Gerard and Meidell, 1993; Graham and Prevec, 1992) currently gives rise to growing interest from biotechnologists. All of these applications will require the production of large quantities of either recombinant proteins or AV stocks. However, so far, no significant research efforts have been directed towards the scale-up of the AV expression system.

Helper-independent AVs were developed in the early 80's for high-level expression of recombinant proteins in human cells. By deleting the E1 and E3 regions

of the adenovirus genome, transcription cassettes up to 7.0Kbp could be inserted in AV (Gluzman *et al.*, 1982). While the deletion of the E3 region only affects the ability of the AV to efficiently propagate in whole animals (Berkner, 1988), the deletion of the E1 region prevents its replication in all mammalian cells (either in vivo or in vitro). However, AV lacking the E1 and E3 regions can be propagated in the human 293 cell line which constitutively expresses the adenovirus E1 polypeptides (Graham *et al.*, 1977, Berkner, 1988). Thus, the AV/293 expression system has a double lock security feature built-in that restricts the propagation of replication defective recombinant viruses to the complementing 293 cell line. Typically, the construction of replication defective AV for recombinant protein production is accomplished by inserting Major Late promoter-based (MLP) expression cassettes in place of

the deleted adenovirus E1 region. The adenovirus MLP is one of the strongest mammalian promoters and its transcriptional activity is responsible for the accumulation of the abundant adenovirus late proteins which represent collectively as much as 30–40% of total cellular proteins in adenovirus-infected cells (Ginsberg, 1984). However, due to the complexity in the regulation of gene expression in adenovirus, the recombinant protein production, using the first generation of expression vectors, has never exceeded 4% of the total proteins (estimated from data in Berkner, 1992). Consequently, the development of the full potential of AV as a high-level expression system has lagged behind other similar expression vectors such as the baculovirus/insect cells system (reviewed in O'Reilly *et al.*, 1992).

Recently, we have reported the construction of a new adenovirus expression vector (pAdBM5) that allows for the production of unprecedented levels of recombinant protein in AV-infected 293 cells (Massie *et al.*, 1994). In 293 cells infected with AV derived from the pAdBM5 transfer vector (AdBM5), the recombinant protein can accumulate at levels up to 10–20% of total cellular proteins (equivalent to 30–60 $\mu\text{g}/10^6$ cells), which makes it the most abundant protein in the infected cells. This yield compares advantageously to established expression systems, such as baculovirus/insect cell (Bac), for the production of non-secreted protein. Massie *et al.* (1994) compared the production of herpes simplex virus ribonucleotide reductase R1 and R2 subunits in both optimized culture of AV/293 and Bac systems. While the R2 subunit was about 5 fold more abundant and active in AV/293 than in Bac infected Sf9 cells, the R1 subunit was produced at roughly similar level in both systems. However, the amount of active soluble R1 obtained from AV/293 was at least 5 times higher than in Bac/Sf9 presumably due to better folding of the R1 protein in 293 cells. In terms of scale-up, the fact that, contrarily to Bac, AV virions remain concentrated within the cell long after yields have reached maximum levels, facilitating virus collection and concentration (Graham and Prevec, 1992) is also an advantage over Bac.

Another scale-up issue was the difficulty to design a large scale unit for the culture of adherent cells. The adaptation of the original 293A (anchorage-dependant) cells to suspension culture was a pre-requisite for the scale-up of the AV/293 system. The 293N3S subline developed by Graham (1987) by passage of the 293A cells through nude mice, was the first subclone of 293 cells successfully adapted to suspension culture. In our

hands however, the 293N3S cells had a relatively long initial lag phase in suspension, a low growth rate, and a strong tendency to clump, even in calcium-free medium. We then tested another subline, the 293S cells (Cold Spring Harbor Laboratories), obtained by gradual adaptation to suspension growth. The 293S cells grew more readily in suspension with no initial lag phase, a doubling time of 24 h and minimal clumping in calcium-free medium. Furthermore, 293S cells produced equivalent level of recombinant proteins compared to 293A (Massie *et al.*, 1994). The 293S cell line was therefore chosen for further process development.

Medium limitation and/or by-product inhibition is an important scale-up problem in animal cell culture in general and the AV/293 system is not an exception. Although 293S cells could reach plateau density of $2\text{--}5 \times 10^6$ cell/mL depending on the culture medium, productive infection with AV was restricted to cell density lower than 5×10^5 cells/mL in batch culture without medium replacement. In this paper, we present a two-step approach, undertaken to improve the volumetric yield of the AdBM5/293S recombinant protein production system with the model protein Protein Tyrosine Phosphatase 1C (PTP1C). This 68kDa enzyme is a highly phosphorylated intracellular phosphatase that plays a crucial role in signal transduction and is a potential target for cancer therapy (Shen *et al.*, 1991). In the first step a medium replacement strategy has been applied, in order to rapidly overcome any medium-related limitation/inhibition problems. We will show the success and the limitations of this strategy. In a second step, an extensive metabolic analysis has been undertaken in order to identify more precisely what was limiting or inhibitory in the medium. This knowledge was then applied to a specific addition and control strategy of the infected culture. This longer procedure has lead to higher volumetric productivity with lower medium expenses.

Materials and methods

Cells, medium and virus

The 293A cells were used for plaque assay. The cells are derived from human kidney fibroblast transformed with Ad5 DNA and express the E1A and E1B proteins constitutively (Graham *et al.*, 1977). 293A were obtained from ATCC and sub-cultured twice weekly in DMEM with 10% fetal bovine serum in 25 cm² T-flasks. The 293S were obtained from Dr. Michael

Matthew (Cold Spring Harbor Laboratories). 293S were kept frozen in liquid nitrogen until used. A fresh cell aliquot was thawed every two months and maintained in 100 mL spinner flask at 37 °C, 5% CO₂ by diluting twice a week to cell densities $1-3 \times 10^5$ cells/mL with complete Joklik + medium (J+) described below.

J+ medium was inspired from Chillakuru *et al.* (1991) who used enriched DMEM for cultivation of vaccinia virus in HeLa cells. It was made of Joklik medium (calcium-free modification of MEM, Sigma) supplemented with 2.5 g/L glucose (total 4.5 g/L, 25 mM, Sigma), 1X MEM essential amino acids (Gibco), 1X MEM non-essential amino acids (Gibco), 1X MEM vitamin solution (Gibco), 0.11 g/L Na.pyruvate (Gibco), 5.7 g/L NaHCO₃ (Sigma) and 2.5 g/L HEPES buffer (Sigma). The mixture was then adjusted to pH=6.75 and filter-sterilized. Calcium-free DMEM (CFDMEM, custom made, Gibco) was also tested. This medium was supplemented to yield a final concentration of 4.5 g/L glucose and 0.11 Na.pyruvate, equivalent to J+. However, CFDMEM was different from J+ as it did not contain any other non-essential amino acids except serine (0.1 mM in J+ vs 0.4 mM in CFDMEM) and its buffering capacity consisted in 3.7 g/L of NaHCO₃. Both media were always completed with 5% iron supplemented bovine calf serum (CS)(Hyclone) and 0.1% (w/v) pluronic F-68 (Gibco) unless otherwise stated.

The replication defective AV, AdBM5-PT, have been constructed in Dr Shen's laboratories, to produce protein tyrosine phosphatase (PTP1C)(Zhao *et al.*, 1993). A stock of the virus has been constituted and used throughout all of the experiments: 6×10^9 cells were infected at a multiplicity of infection (number of viruses/number of cells or MOI) of 1 and harvested 72 hours post-infection (hpi). The cell pellet was then diluted to 10^7 cells/mL with J+ and then freeze-thawed three times to liberate the virus. The stock has then been titrated to 1.2×10^9 pfu/mL by standard plaque assay method.

Culture and infection in spinner flasks

Unless otherwise stated, culture and production runs were done in 100 mL siliconized spinner flasks (Bellco) with 50 mL of cell suspension in a 37 °C, 5% CO₂, humidified incubator. Samples were taken on a daily basis for viable and total cell count and were kept at -80 °C for further analyses. Aliquots of 1×10^6 cells

were centrifuged (13,000 g), the cell pellet was washed twice in PBS and then frozen at -80 °C.

Growth of 293S cells in batch cultures were initiated by inoculating fresh J+ medium with $1-2 \times 10^5$ cell/mL in exponential growth phase.

Production runs were prepared by first centrifuging (600g, 15 min) aliquots of a cell culture in the exponential phase or in the very beginning of the plateau phase. To insure that the viral adsorption phase was identical for each assay, uniform conditions were imposed for the initial incubation of the cell/virus mixture. The cell pellets were then resuspended with the AdBM5-PT virus in either spent or fresh medium at a cell density of 10^7 cells/mL and a MOI of 10 to insure synchronous infection. These concentrated cell/virus suspensions were incubated 2 hours and then diluted at various cell densities with spent or fresh medium. The infected cultures were incubated 3-5 days while samples were taken once or twice daily. For medium replacement experiments, infected cultures were centrifuged at 600g for 15 min and the spent medium discarded and replaced with the same volume of fresh medium. In a few cases, pH was periodically adjusted (2-3 times/day) in spinner flasks by addition of 7.5% NaHCO₃ until the color of the culture returned to 7.1. In spinner flasks, pH was estimated by the medium color compared to standard flasks (red-orange at pH \approx 7.1, yellow at pH \leq 6.5).

Bioreactor description and operation

A 3.5 L bioreactor (Chemap CF-3000 with a CBC-10 control unit) was used with 2.7 L of culture volume in order to scale-up the spinner productions. The tank was equipped with 3 surface baffles to break the liquid surface and increase mass transfer. Mixing was performed with a marine impeller rotating at 100 RPM. The temperature was maintained at 37 °C with a water jacket. D.O. and pH probes (Ingold) were mounted for monitoring and control purposes. The pH was controlled at 7.0 by intermittent addition of 7.5% NaHCO₃ solution. Feed gas composition was regulated by the sequential opening of electro-valves: CO₂ was kept at 7% and O₂ set-point was under the control of dissolved oxygen (DO) in order to maintain DO above 20%. Operation parameters were sent to a Compaq Deskpro PC for data acquisition.

The bioreactor infection protocol was identical to the one used for spinner cultures except that transfer of fluid to and from the bioreactor was achieved through sterile connections instead of under a biological hood.

Analytical methods

Viable and total cells were counted on a haemocytometer. Viability was assessed by dye exclusion using erythrosine B. The 293S cells having the tendency to agglomerate, special care was taken to separate the clumps without affecting viability.

Medium composition was analyzed via HPLC. The various amino acid concentrations were measured by a reversed phase method as described previously by Kamen *et al.* (1991). The glucose and organic acid concentrations were obtained using an Interaction Ion-300 organic acid column (Chemicals Inc) with 0.0033N sulphuric acid as a mobile phase and two detectors: a refractive index detector (model 410, Millipore) and a spectrophotometer detector (model 490, Millipore) at 210 nm.

The oxygen uptake rate was measured using a YSI model 53 biological oxygen monitor, following the protocol provided with the system.

SDS-PAGE electrophoresis of cellular proteins was performed as follows. Frozen cell samples were thawed and diluted to 10^7 cell/mL in extraction buffer (80 mM Tris-HCl pH 6.8, 2% (w/v) SDS and 10% (v/v) glycerol) and then sonicated (Heat Systems-Ultrasonics Inc, model W-375) 5s, 90W. Cell extracts (10 μ L) were diluted with 10 μ L of NOVEX (San Diego, CA) sample buffer, containing 0.5% (v/v) β -mercaptoethanol. The diluted samples were heated at 85 °C for 5 min. and centrifuged 15s in an Eppendorf centrifuge before being loaded on a 8% acrylamide NOVEX precasted gel (10^5 cells per lane). The SDS-PAGE was run for 90 min. at 125 V following the NOVEX procedures.

Protein Tyrosine Phosphatase activity

PTPIC activity was measured according to the method described by Pot *et al.* (1991) with the following modifications. Batch analysis was performed by doing multiple dilutions in 96-well plates where samples were quantified against a PTPIC standard (kindly provided by Dr S. Shen). However, it was found that when diluted in the extraction buffer alone, the activity of the purified enzyme was drastically reduced. In order to stabilize the enzymatic activity, the purified PTPIC was diluted in a cell lysate obtained by adding 400 μ L of the extraction buffer (described below) per 1×10^6 non-infected cell pellet. Samples, stored at -80 °C, were thawed on ice and resuspended at 2.5×10^6 cells/mL in extraction buffer: 25mM Tris-HCl, pH = 7.5, 10 mM

β -mercaptoethanol, 2mM EDTA and 0.5% (v/v) Triton X-100. Aliquot volumes of 1 to 6 μ L were transferred in the 96 well plate followed by 95 μ L of pNPP reagent: 25mM pNPP, 1.6mM DTT, 40 mM MES, pH=5. The plate was incubated at room temperature for 10 min and then 100 μ L/well of 0.2N NaOH solution was added to stop the reaction. The plate was read at 405 nm using a Titertek Multiskan MCC microplate reader. A calibration curve was obtained from dilutions of the standard and the PTPIC content of the samples was calculated from that curve.

The specific PTPIC activity has been verified to be equivalent for cell samples and purified standard. For dilutions of identical activities, the PTPIC band obtained on PAGE for the purified standard was always equal or less than the PTPIC band for a cell sample. The stability of the frozen PTPIC standard was also assessed by series to series reproducibility of the activity calibration curve.

Yield (Y) and specific consumption rate (q) calculations

During growth experiments, the limits of the exponential growth phase were identified by first determining the zone of linear relationship on the plot of the natural log of cell concentration $\ln(X)$ vs time (t). The specific growth rate (μ) was then estimated as the slope of that $\ln(X)$ vs t plot and the doubling time (t_d) was computed: $t_d = \ln(2)/\mu$. The cellular yield per mole of consumed substrate (Y_s) was calculated by dividing the quantity of cells produced by the quantity of substrate consumed during the exponential growth phase, while the product per cell yield was the mole produced divided by the quantity of cell produced during the exponential growth phase. The specific substrate consumption (q_s) rate was obtained by using: $q_s = \mu/Y_s$.

During infection, since cells do not grow significantly, Y and q were calculated differently. Specific substrate consumption rates were estimated during the period of initial linear consumption by dividing the quantity consumed by the time interval and the mean cell concentration during that period. Product yield was obtained by dividing the maximum quantity produced by the total cell concentration at that time.

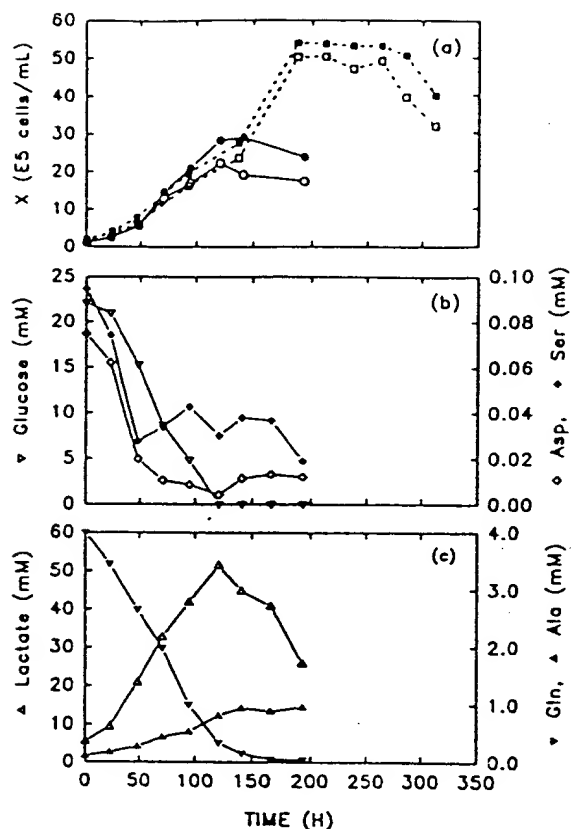


Fig. 1. Typical growth curves of 293S cells in Joklik+ and CFDMEM. (a) \circ , \bullet viable and total cells in J+, \square , \blacksquare viable and total cells in CFDMEM. Key metabolites profiles for cultures grown in Joklik+ medium: (b) ∇ glucose, \diamond aspartate, \blacklozenge serine, and (c) Δ lactate, ∇ glutamine, \blacktriangle alanine.

Results and discussion

Growth kinetic of 293S cells in suspension culture

Typical growth curves for the 293S cells in J+ and CFDMEM, together with the variation of key medium components in J+ is shown in Fig. 1(a) through (c). Viable and total cell densities are shown in Fig. 1(a). 293S cells were inoculated at 0.15×10^6 cells/mL in complete J+ medium. Exponential growth started soon after cell inoculation and was maintained for a period of 3 days with a doubling time of 24 h. The growth was then linear for the next two days followed, at day 5, by a plateau of $2\text{--}3 \times 10^6$ cells/mL. At that point, the viability of the culture did not decrease sharply but rather stayed at the plateau for a few days before declining.

As can be seen in Fig. 1(b) and (c), the growth of 293S cells in J+ was characterized by a substan-

tial consumption of: glucose ($Y_{\text{glucose}} = 10^{11}$ cell/mole or $q_{\text{glucose}} = 29 \times 10^{-14}$ mole.(cell.h) $^{-1}$ in the exponential phase), aspartate ($Y_{\text{asp}} = 7.1 \times 10^{12}$ cell/mole or $q_{\text{asp}} = 0.4 \times 10^{-14}$ mole.(cell.h) $^{-1}$), serine ($Y_{\text{ser}} = 6.7 \times 10^{12}$ cell/mole or $q_{\text{ser}} = 0.43 \times 10^{-14}$ mole.(cell.h) $^{-1}$) and glutamine ($Y_{\text{gln}} = 5 \times 10^{11}$ cell/mole or $q_{\text{gln}} = 5.8 \times 10^{-14}$ mole.(cell.h) $^{-1}$). Although glucose and glutamine were consumed at a higher specific rate, aspartate and serine were depleted first since they are at a much lower concentration in the medium (0.1 mM compared to 25 mM for glucose and 4 mM for glutamine). Ammonia never exceeded 2 mM at which concentration it was tested to be non-inhibiting for 293S growth (data not shown). On the other hand, lactate was the main by-product of the culture ($Y_{\text{lact}} = 2 \times 10^{-11}$ mole produced/cell) while a marginal amount of alanine was produced (1 mM at day 6). Indeed, 293S cells did not oxidize significant amounts of glucose; most of it was used through glycolysis. Lactate has been found to impede 293S cell growth at concentrations of 20 mM or more (data not shown). Consequently, lactate accumulation might have caused the shift from exponential to linear growth around day 3, although depletion of aspartate and serine may also be involved. Lactate accumulated to 50 mM by day 5, at which point glucose was depleted and the cells stopped dividing. Glucose and/or glutamine depletion around day 5 was most probably the cause of the culture entering the plateau phase, after which the cells started consuming lactate.

The 293S cells were also grown in complete CFDMEM (Fig. 1(a)). As can be seen, while the growth kinetics were equivalent in both media up to day 5, beyond that point, cells in CFDMEM kept growing for another 3 days reaching a cell density of 5×10^6 cells/mL. In contrast, cells in J+ entered the plateau phase at this time. A 3 day plateau then occurred before the cell density started decreasing. As described in the previous section, the only difference between CFDMEM and J+ is the lower buffering capacity and the absence of non-essential amino acids with the exception of serine which is four times more concentrated. The need for higher levels of serine and/or the effect of the buffers (especially HEPES) on cell/substrate yields (glucose or glutamine) could explain the better performance of CFDMEM for 293S cell growth.

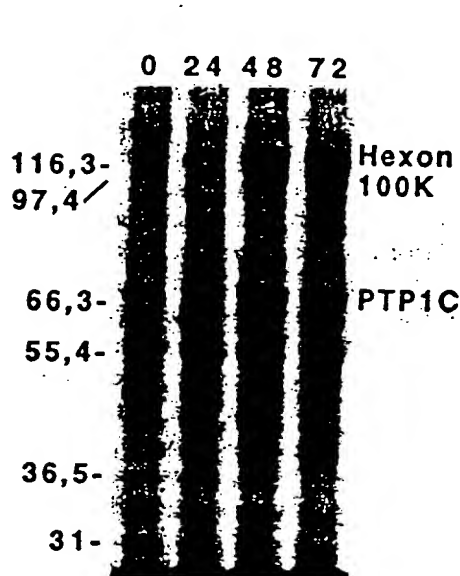
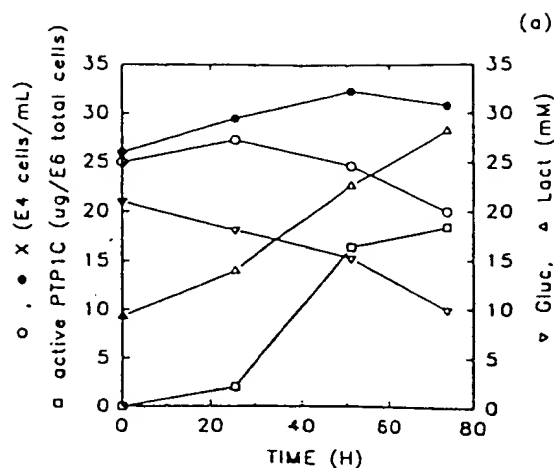


Fig. 2. Typical batch production of PTPIC at low cell density (0.25×10^6 cells/mL) in Joklik+o, \bullet viable and total cells, \square PTPIC, ∇ glucose, \triangle lactate; (b) SDS-PAGE of cell extracts sampled at different time the infection, 10^5 cells per lane: 0, 24, 48 and 72 hpi.

Production of PTPIC in AdBMS-PT infected 293S at low cell density

Fig. 2(a) shows the increase in cell density with time for infected 293S cells seeded at 0.25×10^6 cells/mL. The active PTPIC cell content, glucose consumption as well as the lactate accumulation are also shown. The growth rate for infected cells was close to zero and cell

viability fell gradually during the 3 day production phase. This is typical of a viral infection where the virus utilizes the host's cellular machinery towards the production of its own DNA, RNA and proteins, thus impeding cellular growth and eventually causing cell death. Active PTPIC was produced at a constant rate and reached a level of $18 \mu\text{g}/10^6$ cells 3 days post-infection. Furthermore, as can be seen by SDS-PAGE of the cell extract (Fig. 2b) for samples at 0, 24, 48 and 72 hpi, the PTPIC band at 68kDa followed the same accumulation kinetics as the activity assay. It can also be seen in Fig. 2(b) that PTPIC constituted the most abundant cellular protein, overtaking the hexon and 100K viral proteins.

As for the metabolites' evolution, glucose consumption as well as lactate accumulation were significant but not limiting the culture (Fig. 2a); the other components in the medium were also not limiting (data not shown). This was expected since at low cell density infections, conditions are optimal for production because no limitations and/or inhibitions take place. However, specific consumption rates for glucose, 46×10^{-14} mole.(cell.h) $^{-1}$ and glutamine, 9.4×10^{-14} mole.(cell.h) $^{-1}$ were 60% higher than during growth while the rates for aspartate and serine, $q = 0.25 \times 10^{-14}$ mole.(cell.h) $^{-1}$ decreased by 40% with respect to growth. The significant increase in glucose and glutamine consumption rate, both primary sources of energy, indicate a general acceleration of the cell metabolism during infection. Once again, lactate accumulated at a specific rate twice that of glucose consumption (1×10^{-12} mole.(cell.h) $^{-1}$) implying complete glycolysis.

Production of PTPIC at higher cell densities: effect of medium replacement

A first series of experiments was undertaken in order to precisely establish the potential of the production medium in terms of the maximum cell density at infection that would not impair product yield. 293S cells were infected at different days in a culture and therefore at different densities (day 2, 0.6×10^6 , day 3, 1.3×10^6 and day 4, 1.7×10^6 cells/mL) have been infected and resuspended at their initial cell densities in either (a) their spent medium, (b) fresh medium or (c) fresh medium followed with a medium replacement at 24 hpi. The resulting PTPIC yields at various time points are presented in Fig. 3 (a) through (c) and compared to a culture control infected a low cell density (0.25×10^6 cells/mL). It can be seen in Fig. 3(a) that at 0.6×10^6

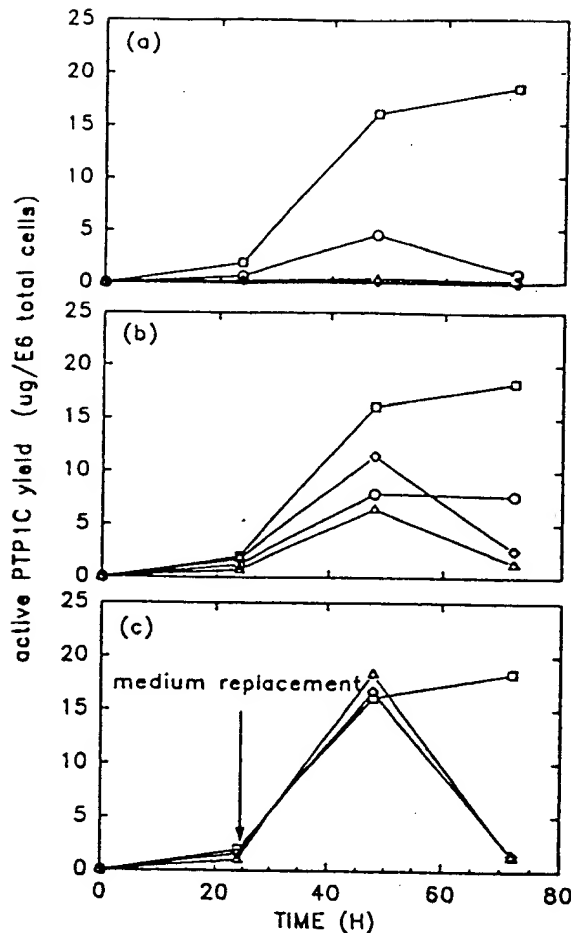


Fig. 3. PTPIC production in J+ at different cell densities. Infected cells resuspended in: (a) spent medium, (b) fresh medium, and (c) fresh medium with medium replacement at 24 hpi. Cell densities at infection: \circ 0.6×10^6 , \diamond 1.3×10^6 and \triangle 1.7×10^6 cells/mL. \square duplicate controls at 0.25×10^6 cells/mL resuspended in spent or fresh medium.

cells/mL or above, no significant amount of PTPIC was produced when the infected cells were resuspended in their spent medium. However, when resuspended in fresh medium (Fig.3b) production regained 50% of the maximum productivity with respect to the control at all cell densities. Furthermore, a second medium replacement at 24 hpi (Fig.3c) allowed for a sustained maximum specific productivity, even at the highest cell density (initial 1.7×10^6 led to a final 2.2×10^6 cells/mL).

These results clearly establish the existence of a substrate limitation and/or a by-product inhibition at high cell densities, a problem which can be partially remediated by an initial cell resuspension in fresh

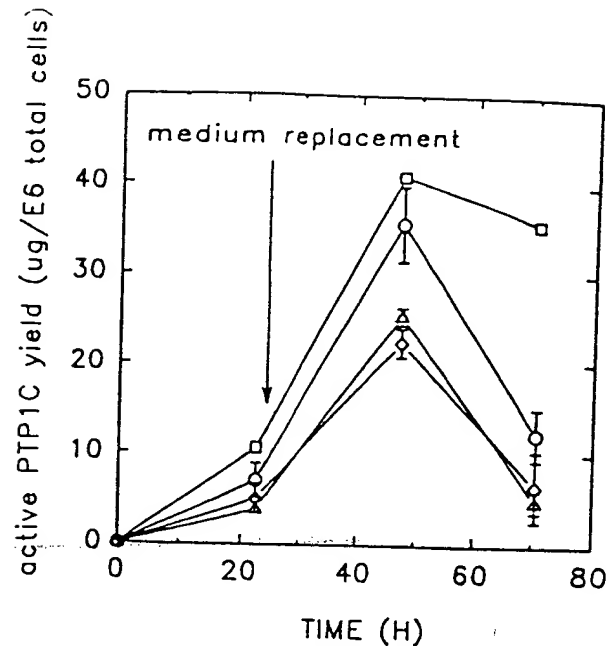


Fig. 4. PTPIC production in fresh J+ at different cell densities with one medium replacement at 24 hpi. \circ 2×10^6 , \diamond 3×10^6 , \triangle 4×10^6 cells/mL. \square control at 3×10^5 (without medium replacement). Means of duplicates are represented (\pm S.D.).

medium (Fig.3b) and completely restored with a medium change at 24 hpi (Fig.3c), resulting in a maximum productivity comparable to optimal low cell density infection. It also establishes that the cell culture growth stage (from early exponential at day 1 to beginning of plateau phase at day 4) do not influence protein production since cells infected in their 4th day of culture produced as much PTPIC as one day old infected culture, as long as the medium is not limiting and/or inhibiting. However, this medium replacement strategy was apparently only effective for a period of 24 to 48 h since the PTPIC activity decreased abruptly at 72 hpi. Analysis of total cell protein by SDS-PAGE showed that the loss in activity at 72 hpi was not concurrent with an equivalent loss in total PTPIC content (data not shown). The decrease in PTPIC activity observed at 72 hpi was therefore not due to protein degradation, but rather to an unknown mechanism such as protein aggregation, as previously observed for the HSV R1 subunit expressed with a similar AV (Massie *et al.*, 1994) or a major change in the phosphorylation state of the protein. Resolving this issue will require further investigation.

In order to evaluate the limit of this initial and 24 hpi medium replacement strategy, PTPIC yield was tested following infection of 293S cells at 2, 3 and

4×10^6 cells/mL. As presented in Fig. 4, at 48 hpi, the yield of PTP1C for infection at 2×10^6 cells/mL ($35 \pm 4.3 \mu\text{g}/10^6$ cells) was not significantly different from the control ($40 \mu\text{g}/10^6$ cells), while for higher cell densities yields were 40% inferior to the control. Past 48 hpi, the active product yield fell to zero for all the experiments at high cell densities while the low density control remained constant. These results show that 2×10^6 cells/mL is the maximum cell density at which daily medium replacement with J+ medium allows for the maintenance of maximum specific productivity. At cell densities higher than 2×10^6 cells/mL, volumetric productivity as well as specific product yield per cell decreases thereby increasing production and purification costs.

Production of PTP1C at high cell density in a 3L bioreactor

Since CFDMEM was more efficient for 293S growth than J+, its performance during an infection was tested. However, a preliminary experiment comparing both media for an infection at high cell density showed that CFDMEM acidified more rapidly than J+, which was not the case during cellular growth. At 72 hpi, in cell cultures infected at 1.3×10^6 cells/mL in fresh medium with a medium replacement at 24 hpi, the pH dropped below 6.5 (yellow medium) in CFDMEM while pH was roughly equal to 6.8 (orange medium) in J+ (data not shown). As a result, the PTP1C activity was also lower in CFDMEM ($20 \mu\text{g}/10^6$ cells) than in J+ ($32 \mu\text{g}/10^6$ cells) at 48 hpi.

In order to assess the correlation between active PTP1C production and pH as well as the scalability of the process, an infection experiment was performed in a pH-controlled 3L Chemap bioreactor. The results are shown in Figure 5 for a culture infected at 2×10^6 cells/mL (MOI=10) in CFDMEM. The bioreactor was compared to infected cell controls: two 50mL spinner flasks initially taken from the bioreactor, one with and the other without periodical pH adjustment.

The PTP1C specific productivity was equivalent in the bioreactor and in the pH controlled spinner with a peak of $45 \mu\text{g}$ active PTP1C/ 10^6 cells at 48 hpi, followed by a slight decrease in the active PTP1C concentration. By contrast, in the spinner flask without pH control, the accumulation of active PTP1C stopped at 30 hpi with a peak of $30 \mu\text{g}$ active PTP1C/ 10^6 cells, followed by a rapid reduction in activity, falling close to zero by 52 hpi. A correlation between the PTP1C activity loss and pH decrease was observed. While at

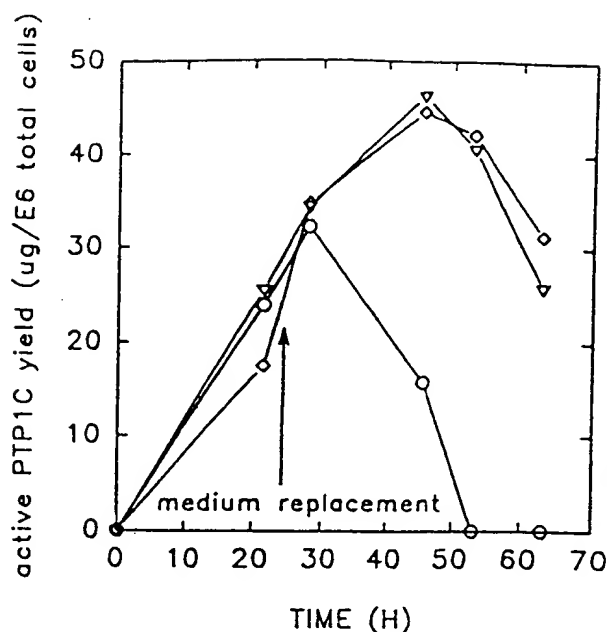


Fig. 5. Production of PTP1C in bioreactor at 2×10^6 cells/mL with fresh CFDMEM and a medium replacement at 24 hpi. ◇ bioreactor, ▽ spinner control with periodical pH adjustments, ○ spinner control without pH adjustments.

48 hpi the medium was already yellow in the non-controlled spinner ($\text{pH} \leq 6.5$), the medium was still red-orange in the bioreactor and the pH adjusted spinner ($\text{pH} \approx 7$). However, the decrease in pH does not fully explain the PTP1C activity loss since the PTP1C activity also decreased slowly in a pH controlled environment past 48 hpi. The pH control only delays the lytic process that inevitably takes place during adenoviral infection, while permitting the maximum product yield to be attained.

These results show that it is possible to maintain maximum specific production rate at high cell densities, by medium replacement at 0 and at 24 hpi, in a pH controlled culture at the 50 mL spinner flask scale as well as 3L bioreactor scale. Although peak product yield varied among the experimental runs, one can expect to obtain 40 to $45 \mu\text{g}$ PTP1C/ 10^6 cells, equivalent to 15% of the total cellular protein content (based on $300 \mu\text{g}$ total protein/ 10^6 cells) or 90 mg PTP1C per Litre of culture (at 2×10^6 cells/mL) compared to 13.5 mg/l for 0.3×10^6 cells/mL. These figures are comparable to productions of Herpes Simplex Virus ribonucleotide reductase subunits R1 and R2 obtained previously in our lab with other AdBM5 AV's (Massie *et al.*, 1994). Although this process is very effective with respect to cell yield and protein purification for

an intracellular product, it is not optimal in terms of medium expenses. In fact, with two medium changes, the yield of product per spent medium is equivalent to 30 mg PTPIC per L of medium (at 2×10^6 cells/mL) which is not much higher than 15 mg/L of medium for productions at 3×10^5 cells/mL without medium replacement. We then turned our attention to the analysis of key metabolites in order to improve yield based on spent medium.

Analysis of key metabolites during the infection phase

Samples from the infection of 1.3×10^6 293S cells/mL in fresh J+ medium without medium replacement at 24 hpi (for which PTPIC yield has been presented in Fig. 3b) have been analyzed for their content in glucose, organic acids, and amino acids. In this experiment, PTPIC was only 60% the level of its maximum specific activity due to limitation of nutrients and/or inhibition of by-products. The results are presented in Fig. 6.

The total cell density increased from 1.3×10^6 to a mean value of 1.9×10^6 cells/mL during the first 24 h and remained constant thereafter. This slight initial increase of about 20% in cell count is routinely observed for infected culture. However, the maintenance of viability up to 96 hpi is peculiar to infection in limiting and/or inhibiting environment. This could be explained by the fact that under sub-optimal conditions of infection, the overall cycle of virus reproduction would occur at a lower rate, thereby reducing the infection stress on the cell which in turn would result in a prolonged viability.

Glucose was completely depleted before 48 hpi. Based on the first 24 hour period, the specific glucose consumption rate, was 38×10^{-14} mole glucose.(cell.h) $^{-1}$ which is a 30% increase compared to q_{glucose} during cell growth. However, glucose consumption rate was reduced by 17% compared to infection at low cell density. Furthermore, while glucose was totally transformed into lactate during growth and infection at low cell density, only 45% of it was metabolized through glycolysis during the infection phase at high cell density; 22mM of glucose yielding only 20mM of lactate instead of 44mM for a complete glycolysis. It appears that while the general metabolic activity was higher compared to growth phase, in this case there was a glucose limitation, thereby reducing glucose consumption rate as well as lactate production rate. This in turn should increase the cellular oxygen requirement. The specific oxygen consumption rate

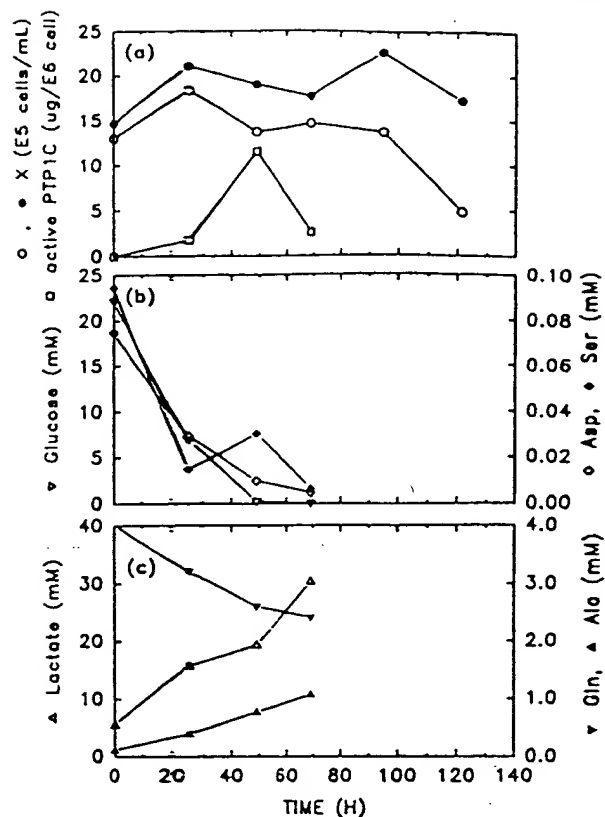


Fig. 6. Key metabolites evolution in a batch production of PTPIC in fresh J+ at initial cell density of 1.3×10^6 cells/mL: a) \circ , \bullet viable and total cells in J+, \square PTPIC yield; b) ∇ glucose, \diamond aspartate, \blacklozenge serine, and c) \triangle lactate, ∇ glutamine, \blacktriangle alanine.

(q_{O_2}) has been measured in growth phase as well as during infection. Indeed, the average value obtained during infection, $q_{O_2} = 16 \times 10^{-14}$ mole O_2 .(cell.h) $^{-1}$ was twice as high as for exponential growth, $q_{O_2} = 8 \times 10^{-14}$ mole O_2 .(cell.h) $^{-1}$. This is consistent with a significant increase in metabolic rate during the infection phase compared to the growth phase and a reduced glycolysis rate with respect to nonlimiting medium conditions.

During infection, aspartate and serine specific consumption rates were low ($\approx 0.2 \times 10^{-14}$ mole.(cell.h) $^{-1}$ for both), but, given the high cell density, were rapidly depleted by 48 hpi. Glutamine was consumed (2.2×10^{-14} mole.(cell.h) $^{-1}$) but not depleted and alanine was produced (1×10^{-14} mole.(cell.h) $^{-1}$). These specific consumption rates were smaller than those obtained for infection at low cell density and exponential growth. This might be due to glucose limitation. However, aspartate and serine, although depleted, were not limiting. Indeed, compar-

isons of PTPIC production in J+ and CFDMEM at high cell densities were not found to be significantly different, even though CFDMEM did not contain aspartate but four times the serine concentration of J+. In other production runs, 6mM glutamine (instead of 4mM) as well as 10% serum addition (instead of 5%) have also been tested, but did not give higher productivities (results not shown). However, a 50% drop in PTPIC production was observed in absence of serum compared to the usual 5% CS.

In summary, in serum supplemented culture, the production of PTPIC with the AdBM5/293S system in CFDMEM was mainly limited by glucose depletion and inhibited by the pH drop caused by lactate accumulation.

Effect of specific additions during a production run

In order to apply and verify the above results, PTPIC production was compared in a glucose addition vs a medium replacement experiment. A culture at 1.5×10^6 cells/mL was infected, resuspended in fresh CFDMEM with 2.5 g/L HEPES and aliquoted into three 50mL spinner flasks. In the first spinner the culture was centrifuged and the medium replaced with fresh CFDMEM + HEPES at 24 hpi. In the other two, 0.5 mL of a 200 g/L glucose solution was added to the culture at 24 hpi (+2 g/L, or 11 mM glucose addition). In one of these pH was periodically adjusted.

Figure 7 shows that, PTPIC production followed a similar profile in both the medium replacement and the glucose addition experiment where pH was controlled. The two feeding strategies yielded a maximum active PTPIC content of $25 \mu\text{g}/10^6$ cells at 36–48 hpi. Since it has been shown that at high cell density without medium replacement or glucose addition, active PTPIC yield declined after 24 hpi, it is clear that glucose addition is responsible for sustained PTPIC production, equivalent to production with medium replacement. It therefore confirms that glucose is most probably the major limiting substrate of PTPIC production.

In the third spinner (glucose addition without pH control) the PTPIC activity decreased linearly after 24 hpi and was absent by 48 hpi. This severe drop in activity is again correlated with a decrease in pH; indeed, the medium was already yellow at 36 hpi or earlier ($\text{pH} \leq 6.5$) in the glucose addition spinner without pH control while it was maintained around red-orange ($\text{pH} \approx 7$) in the other spinner with glucose addition. The exclusive relation between pH and PTPIC activity

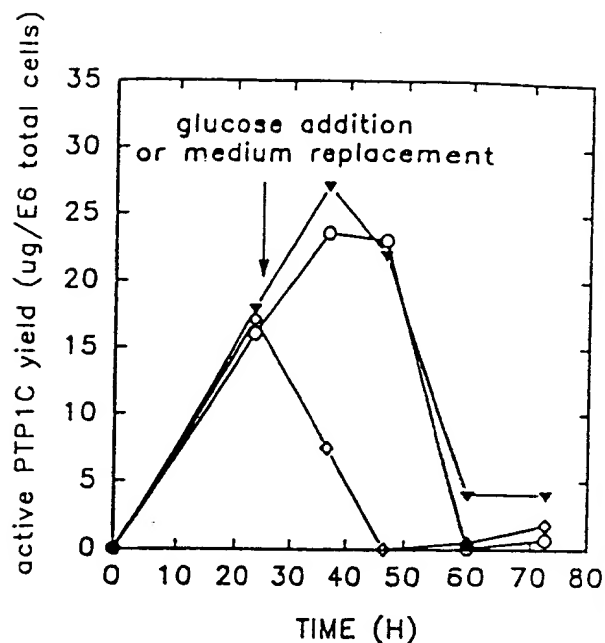


Fig. 7. Production of PTPIC in fresh CFDMEM at 1.6×10^6 cells/mL. ○ medium replacement at 24 hpi, ▼ glucose addition with periodical pH adjustments, ◇ glucose addition without pH control.

was confirmed by lactate analysis since lactate concentrations attained equivalent level (40–50 mM) in both glucose-supplemented spinners (data not shown). It is therefore the pH decrease as such and not lactate production, that has a negative effect on active PTPIC yield. This is encouraging since pH is easier to control than lactate production.

Conclusion

In this paper we have presented the first results concerning the scale-up of a high-level recombinant protein production AV/293 system. The 293S cells have been shown to be able to grow to plateau cell densities of 5×10^6 cells/mL in calcium-free DMEM. With an initial and a 24 hpi medium replacement, the specific PTPIC yield could be maintained at its maximum level up to infected cell densities of 2×10^6 cells/mL. Under these conditions, volumetric productivities of 90 mg/L could be attained. At an infected cell density of 1.6×10^6 cells/mL, the replacement of the 24 hpi medium change by a 2 g/L glucose addition, together with periodical pH adjustments, allowed the same specific productivities, but at lower medium expenses.

It is expected that glucose fed-batch in pH-controlled bioreactor will further improve these performances.

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References

- Berkner KL (1988) Development of adenovirus vectors for the expression of heterologous genes. *Biotechniques* 6:616-628.
- Berkner KL (1992) Expression of heterologous sequences in adenoviral vectors. *Curr. Top. Microbiol. Immunol.* 158:39-66.
- Chillakur RA, Ryu DDY, Yilma T (1991) Propagation of recombinant vaccinia virus in HeLa cells: Adsorption kinetics and replication in batch cultures. *Biotechnol. Prog.* 7: 85-92.
- Gerard RD and Meidell RS (1993) Adenovirus-mediated gene transfer. *Trends Cardiovasc. Med.* 5:171-177.
- Ginsberg HS (ed.) (1984) *The adenoviruses*. Plenum Publishing Corp., New-York.
- Gluzman Y, Reichl H and Solnick D (1982) Helper-free adenovirus type 5 vectors. In *Eucaryotic viral Vectors*, pp. 187-192. Edited by Y. Gluzman, New York: Cold Spring Harbor Laboratory.
- Graham FL, Smiley JR, Russell WC and Nairn R (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36:59-72.
- Graham, FL (1987) Growth of 293 Cells in Suspension Culture. *J. gen. Virol.*, 68:937-940.
- Graham FL and Prevec I (1992) Adenovirus-based expression vectors and recombinant vaccines, p.363-390. In RW Ellis (ed.), *Vaccines: new approaches to immunological problems*. Butterworth-Heinemann, Boston.
- Kamen A, Tom R, Caron A, Chavarie C, Massie B, Archambault J (1991) Culture of insect cells in a helical ribbon impeller bioreactor. *Biotechnol. Bioeng.*, 38:619-628.
- Massie B, Dionne J, Lamarche N, Fleurent J, Langelier Y (1994) Improved adenovirus vector produces herpes simplex virus ribonucleotide reductase R1 and R2 subunits more efficiently than baculovirus vector. Submitted to *Bio/Technology*.
- O'Reilly KR, Miller LK and Luckow VA (1992) *Baculovirus expression vectors*, a laboratory manual. W.H. Freeman and Company, New-York.
- Shen S-H, Bastien L, Posner BI, Chrétien P (1991) A protein-tyrosine phosphatase with sequence similarity to the SH2 domain of the protein-tyrosine kinases. *Nature*, 352: 736-739.
- Zhao Z, Bouchard P, Diltz CD, Shen S-H and Fischer EH (1993) Purification and characterization of a protein tyrosine phosphatase containing SH2 domains. *J. Biol. Chem.* 268:2816-2820.

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Overview of Cell Culture Systems and their Scale-up

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1. INTRODUCTION

In this section of the book an attempt is made to review the many culture systems which have been developed in response to the need for mass cultivation of cells and their products. Each contribution is presented by an expert on that particular culture who will underline the advantages, and limitations, of using a particular technique or reactor. The range of systems

now available seems at first sight to be enormous, but in fact many are divergences of a common technical development, often made 20–30 years ago. Also, many distinctively different systems do run on the same set of conceptual factors. One such parameter is that if cells are to be maintained at a high density and in a controlled environment, then a medium perfusion system has to be used. The problems to be overcome in scale-up are well known. They include maintaining a proper supply of nutrients (including oxygen) without causing any physicochemical damage to the cells and, at high densities, removing toxic metabolites. The problem is one of transfer between the environment and the cell. The various means of solving this problem have given rise to the diversity of culture systems that are currently in use. For anchorage-dependent cells there is the additional problem of increasing the surface area for cell attachment and this again has been solved in many different and ingenious ways.

In this overview chapter, the general problems involved in scale-up are discussed and a historical development of culture systems is presented. This has not only been done out of interest to show the developmental themes which have led to current culture reactors, but also because some of the original or "old" technology used ingenious solutions to the problem which may stimulate today's innovators of culture reactors. The range of available equipment and techniques is confusing for newcomers who have to decide which system will best fit their needs, if indeed they can be persuaded to go beyond multiple roller bottles or low-density suspension cultures. Resources do not usually allow a multiple choice to be made and it is difficult to evaluate the relative performance of competing culture types and to decide whether inherent complexities would make the system risky for a commercial process. Therefore, in this section an attempt is made to give as objective a summary as possible of the strengths and weaknesses, the scale-up capabilities, and the potential applications of the culture systems reviewed.

It is recognized that no single cell-culture system can be universally used, owing to the variability in cell types, product expression kinetics, and process requirements (listed in Table I). Suspension culture is the scale-up method of

TABLE I Factors Affecting the Design of Cell Reactors and Production Processes

	Factors	Parameters
Growth mode	Suspension	Differences in fragility
	Anchorage	Differences in adhesion
Production	Product expression	Growing or stationary cells
	Product type	Intracellular/extracellular/yield
	Product concentration	Feedback inhibition
	Culture type	Batch, fed-batch, continuous
Downstream processing	Product concentration	Ultrafiltration?
	Batch or continuous	

7. Cell Culture Systems and their Scale-up

TABLE II Comparison of Substrate-attached and Suspension Culture Systems

Attached systems	Suspension systems
Easy to change medium	Easy and cheap to scale-up
Easy to perfuse	Unit scale-up to high volumes
Easy to change medium: cell ratio	Utilizes less space
Products often expressed better	Easy to monitor and control
More flexible (all cells)	Cells can be sampled
(Microcarrier culture has the advantages of both systems)	

choice because environmental monitoring and control allows homogeneous and near-linear increase in volume. However, many commercially important cell lines either will not grow in suspension culture, or have a significantly lower productivity, or express products with altered properties in suspension when compared to growth in anchorage-dependent cultures (87). Although suspension culture technology is predominant, and every means of using such cells is being exploited, it is still very important to increase the productivity and unit size of anchorage-dependent reactors. The comparative merits of suspension and anchorage-dependent cells are summarized in Table II. Similarly, every effort is being made to construct continuous, as opposed to batch, processes as a more cost-effective methodology (to avoid repetitive production of cell seed with the accompanying quality-assurance workload, and to avoid equipment downtime and turn-around costs). Again, this is not always possible, e.g. in production of lytic viruses, but the change in emphasis over the last few years on producing recombinant rather than native products encourages the use of continuous processes, even for vaccines.

These factors have to be taken into account when considering the various culture options reviewed on the following pages.

2. SCALING-UP

Scaling-up from small laboratory flasks (static or spinner) in a unit rather than multiple process has always been a prime objective for cell culturists. Initially it was to meet the demands of virologists both for research and the manufacture of vaccines. Currently there is a wide range of molecules that can be economically developed only if efficient large-scale culture methods are used. Animal cells, in comparison to bacteria, have low productivity as a consequence of a slow growth rate (doubling time of 12–24 hr compared to 0.3–3 hr for bacteria). Thus, on a cell weight basis (g biomass litre⁻¹ hr⁻¹) bacteria are 60- to 200-fold more efficient. In addition, animal cells need far more complex and expensive growth media (31) and more critical environmental control and process handling (to avoid the high risk of microbial

TABLE III Recombinant Animal Cell Products

Viruses	H1N1, HSV 1 & 2, influenza, CMV, EBV, rabies, HIV, FMDV, LaS, JEV, JE, RSV, VSV
Blood products	Factor VIII, Factor IX, Protein C, immunoglobulins
Hormones	hGH, hCG, insulin, erythropoietin, relaxin, LH
Others	t-PA, α & β -IFN, IL-2, PDGF, MCAB (e.g. OKT-3)

* Expressed via Vaccinia virus.

contamination, for example). Animal cells are only considered for an industrial process when there is no alternative, i.e. when prokaryotic and other eukaryotic systems, even after recombinant DNA technology, cannot produce the required product in the correct configuration or with freedom from toxic components. Thus, comparisons between bacterial and animal cells are unproductive—the challenge is to increase the efficiency of existing cell systems. This has already been significantly achieved by means of hybridization techniques (DNA and cell) and by optimizing the environment in which cells grow. There already exist many recombinant products from animal cells (Table III). This chapter, however, is concerned with the increased productivity that can be achieved with the correct choice of culture reactor.

The need to achieve higher productivity is exemplified by the data in Table IV (45), which give an indication of the culture volumes needed to produce clinical doses of many important biologicals.

The basic problem in achieving these aims is the relative fragility of cells to mechanical stress brought about by stirring, sparging, etc. As only low-speed, non-turbulent, stirring can be used, mass transfer rates are low and means of

TABLE IV Culture Requirements for Producing Therapeutic Doses of Animal Cell Products*

Product	Cell requirement per dose	Culture volume (litres)
Polio	2×10^4	0.0001
Rabies	4×10^4	0.005
HSV	2×10^7	0.03
FMDV	2×10^7	0.01
IFN (anti-viral)	10^6 day^{-1}	0.1
IFN (anti-tumour)	$5 \times 10^6 \text{ day}^{-1}$	0.5
t-PA	$>10^{10}$	1–10
MCAB	10^{12}	100
UK	10^{12}	500

* Data based on Kaibinger and Blum (3).

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aeration and maintaining optimum nutritional conditions in a non-damaging manner are difficult. There are four major areas in which key developments have occurred to improve cell culture performance: mixing, aeration, perfusion and substrate (for anchorage-dependent cells). These factors are all closely interlinked and should not be considered in isolation, but they do allow individual innovations to be conveniently described.

One major development which has evolved as a combined result of these innovations is the ability to maintain cells at high densities (over 10^8 cells ml^{-1}). Currently there is a choice between volumetric scale-up (increasing size whilst maintaining the same process intensity) and density scale-up (increasing the process intensity in relatively small volumes). The ideal which is being strived after is a combination of these techniques, and assessments are being made of all current working systems to find the technology with the potential to achieve this.

2.1. Relative Merits of Process Intensity

In terms of cell density within body tissues and organs, a dense cell population would be in the order of $1\text{--}2 \times 10^8$ cells cm^{-3} . In a culture reactor, assuming a spherical cell of $12 \mu\text{m}$, a dense culture would be in excess of 5×10^8 cells ml^{-1} (89). This then is the upper limit that can be realistically aimed for; but can it be achieved? The supply of oxygen is perhaps the most critical parameter. It has been estimated that a 100 g organ contains 2×10^{11} cells and, as the average blood flow through tissues is $100 \text{ ml g}^{-1} \text{ min}^{-1}$, then cells *in vitro* have a supply of 3.6×10^{12} g oxygen $\text{cell}^{-1} \text{ min}^{-1}$ (89). This is within the range found for cells in culture (86). Blood delivers oxygen to cells through an extensive capillary system (calculated as having a total surface area of $1.9 \times 10^4 \text{ cm}^2/\text{kg body weight}$). This can be mimicked in cell cultures with the use of fibres or thin-walled vinyl tubing. Calculations on how close together these "artificial capillaries" have to be is based on observation as five cell diameters (3), or computer models of oxygen diffusion rates which range from 10–30 cell diameters (89). Thus, a gap of 100–300 μm between oxygen sources should be adequate to maintain dense cells.

It is possible for cells to exist in small-scale culture in dense masses, but scale-up has to be by multiplicity. However, before committing too much of one's developmental resources to determining whether these systems will scale-up as unit processes, the advantages and disadvantages should first be assessed (Table V). The argument that, since a 1-litre culture supports 5×10^8 cells ml^{-1} , then this is equivalent to a 500–1000-litre reactor of conventional cell density ($2\text{--}5 \times 10^4$ cells ml^{-1}), and that thus there is a reduced need for large volumes, is partly over-ridden by the requirement for a large-volume

TABLE V. High-density Culture Systems

Advantages	Disadvantages
Smaller reactor volume	Volumetric scale-up difficult
More concentrated product	Mass transfer problems
Reduced serum requirement	Sterilization problems
Long run lengths	Higher degree of control sophistication needed

reservoir. A given volume of medium is capable of yielding a finite number of cells—for Eagle's MEM this is $2-4 \times 10^5$ cells ml^{-1} in a closed batch system and $1-2 \times 10^6$ cells ml^{-1} in an efficiently controlled recirculating perfusion system (31). Thus a 1-litre high-density culture theoretically needs a 500-litre reservoir. In fact, with a controlled sequential flow of medium from preparation through to product extraction, this value can be halved. In addition, a great saving in space can be obtained now that medium can be supplied in plastic bags (similar to blood transfusion bags) in units up to 500 litres (116). However, these calculations are based on the assumption that product expression per cell is the same in both dilute and dense systems. This is hardly ever true and the fact that one usually gets diminishing returns during scale-up still remains a prime problem. There are many environmental factors that contribute to this phenomenon (e.g. end-product repression and toxic metabolites), but there are also many factors intrinsic to the cell which can only be resolved by careful comparative studies of cell physiology at low and high densities. Proximal contact, together with chemical and neuronal signals, are the main regulatory agencies in animals (29) and the effect of close contact on surface receptors has to be established in dense cell culture.

Facilitated downstream processing owing to a high product concentration is often quoted as one of the advantages of high-density culture. However, for a particular product, one has to decide whether it is easier to concentrate a dilute supernatant or overcome the problem of cell debris contaminating a concentrated supernatant. This example serves as a warning that there are two sides to many of the arguments for and against a particular culture concept and one should not get carried away with intellectual considerations. Undeniably on the plus side for dense cultures is the fact that the requirement for expensive medium components (e.g. serum, growth factors) is significantly reduced (10, 84).

The history of using animal cells for the manufacture of pharmaceuticals has been typified by the concern not to use a transformed or malignant cell, and to prove that the cell line in use has not undergone such a transformation. One requirement was that human diploid cells must remain as a monolayer, i.e. multilayering was taken as a sign of such a change. It is fortunate that

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Biological Control Agencies are able to relax this requirement, and even permit the use of continuous (heteroploid) cell lines (e.g. Vero) owing to improved biochemical techniques for assessing the biological hazards in the system. This change in attitude is very important for the future of dense cell systems as substrates for therapeutic, rather than just diagnostic, products (108).

22. Mixing

This topic has been covered in Chapter 6. The first stirred cultures relied on a magnetic bar which gave laminar mixing and became scale-up limiting at quite low volumes (2-5 litres). Modifications to increase the surface area of a bar magnet by attaching various designs of "paddle" (27, 30, 96, 111) allow a greater mixing capability without an increase in stirrer speed or, more importantly, to the tip velocity of the impeller (which should not exceed $1.5-2 \text{ m s}^{-1}$). The aim is to achieve non-turbulent, streamlined bulk flow patterns within the culture fluid so that mechanical stress damage is minimized. Turbine impellers are damaging to many cell types and the marine impeller has become the configuration of choice. Modifications to the marine impeller have subsequently been made to increase mixing efficiency at low speeds (119). Other mixing concepts that have been successfully used are the Whorl mixer (20), airlift (43, 44), pump (Celligen fermenter) (75), and internal loop (58). All these systems are diagrammatically summarized in Fig. 1.

22.1. Airlift Reactors

Airlift, as an alternative to standard stirred-tank reactors, has been gaining popularity because of its gentler mixing action and suitability for shear-sensitive cells (5, 43). It works on the principle that the gas mixture introduced into the base of the draft tube within the culture establishes a circulation of medium owing to the density differential between the oxygenated air (in the draft tube) and the less oxygenated air in the outer zone. Oxygen levels and pH can be controlled by varying the composition of the gas mixture. The critical factors are the reactor height, aspect ratio, and gas flow velocity. The optimum configuration for mixing is to have the draft tube diameter essentially the same as the total outside (downflow) diameter, and the most efficient gas velocity is $0.5-1.5 \text{ cm s}^{-1}$ (79). In practice, gas flow rates (AFR) of about 300 ml min^{-1} are employed. Scale-up is relatively simple as it is directly linear and 1000-litre reactors are in commercial use (135, 6). It having been established that cells can withstand cyclical exposure to hydrostatic pressures of 35 psi, plans are well advanced in commissioning a 5000-litre vessel. In some respects it is easier to scale-up, as increases in height improve the mixing times

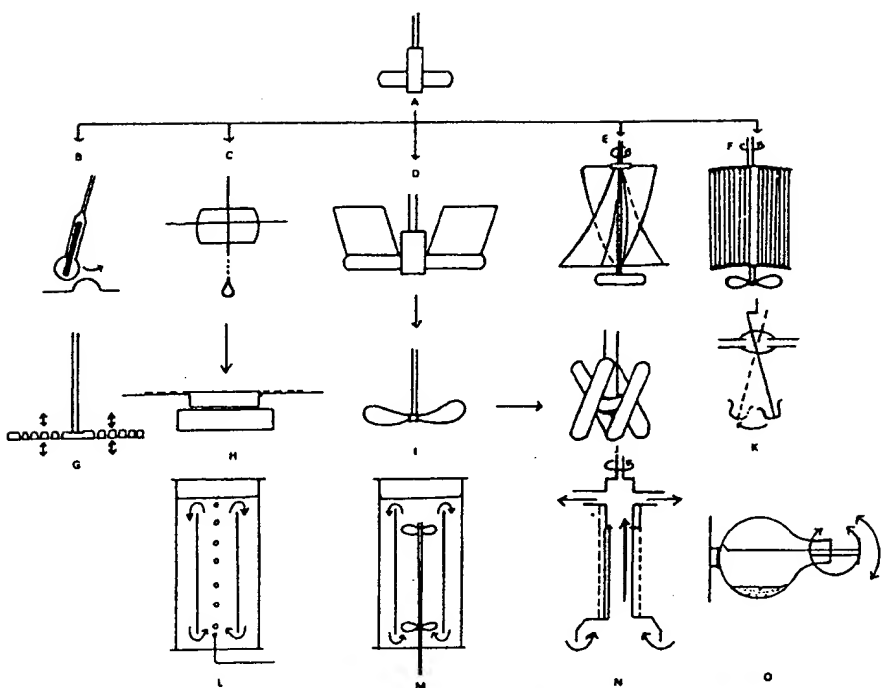


Fig. 1. Agitation/mixing systems used for animal cell cultures. A, magnetic spinner bar; B, MCS system (120); C, cylindrical surface stirrer (15); D, increased surface area spinner; E, sail agitator (16); F, membrane basket agitator (59); G, vibromixer (20); H, surface stirrer BKC6 (12); I, marine impeller; J, cell accelerator (119); K, skull stirrer (2); L, airlift; M, internal loop (18); N, celligen (77); O, ZKA bioreactor (114).

and mass transfer rate. Aspect ratios of between 6:1 and 12:1 are normally used.

The performance and productivity of the airlift system are similar to that of conventional stirred reactors, i.e. $2-3 \times 10^6$ cells ml^{-1} in the batch mode, with the possibility of using continuous or semi-continuous batch feed and perfusion options. It must be considered a low-process-intensity system with no obvious route to significantly increasing cell density, other than an external loop through a filtration unit and returning the cells to the airlift vessel. Although scale-up is straightforward, it does have the disadvantage of being dimensionally linear, requiring considerable vertical space.

The main advantages of airlift over stirred reactors, besides its ease of scale-up and suitability for shear-sensitive cells, is that no moving parts and mechanical seals are needed. This not only means less sophisticated engineering design, but it also eliminates a potential source of contamination. Also, the system makes it easy to meet the required oxygen transfer rates and it requires less power input than other types of culture.

2.3. Aeration

It has long been recognized that bubbling (sparging) in a cell culture vessel causes damage to many cell lines unless it is carried out at very low rates. The general guidelines for sparging are to use large-diameter bubbles (1-3 mm) at a low flow rate ($5-10 \text{ cm}^3 \text{ l}^{-1} \text{ min}^{-1}$). More recently it has been suggested that bubble damage occurs at the surface of the culture during bubble disengagement (13, 36) but that this can be minimized by increasing the aspect ratio of a culture vessel to 6-12:1 in order to reduce the residence time of cells at the surface. This is the basis of the airlift reactor. In addition, the inclusion of Pluronic F-68 (0.01%) very effectively protects cells against bubble damage (13, 36).

Small-scale cultures rely on oxygen transfer through the air-medium interface (surface aeration). However, the relatively low solubility of oxygen (7.6 mg ml^{-1}) and diffusion rates (static cultures) or mixing efficiency (stirred cultures) causes oxygen to become a limiting factor during scale-up (often at 5-litre scale). This is despite keeping a low aspect ratio of 1-2:1. Considerable efforts have been made to supply adequate oxygen in a non-harmful manner

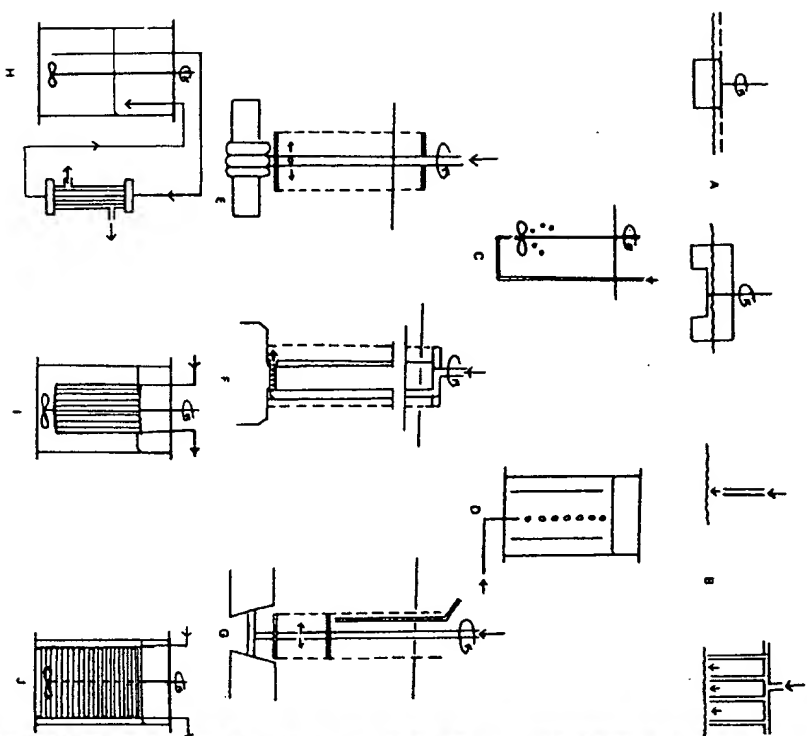


Fig. 2. Systems for aerating animal cell cultures. A, surface aerators (40, 121); B, surface aerators by air jets (100); C, sparging; D, air lift; E, caged aerator (103); F, celligen (75); G, spin oxygenator (35); H, external loop oxygenator; I, membrane basket (69); J, membrane tubing oxygenation (35).

to cell cultures in order to allow scale-up in volume and intensity. The efficiency of surface aeration can be increased at least 4-fold with the use of a surface aerator (40), or by injecting air through multiple nozzles placed immediately above the medium surface (100). Examples of the many alternative methods used to increase aeration are shown diagrammatically in Fig. 2. One method is to circulate the medium from the culture through an

7. Cell Culture Systems and their Scale-up

TABLE VI Aeration Methods of a 39-litre Stirred Bioreactor

Aeration method	O ₂ (mg l ⁻¹ hr ⁻¹)	No. cells/ml × 10 ⁶ supplied*
Air (10 ml l ⁻¹ min ⁻¹ , 40 r.p.m.)	0.5	0.08
Surface aeration	4.6	0.76
Direct sparging	3.0	0.40
Spin filter sparging	12.6	2.10
Perfusion (1 volume hr ⁻¹)	15.9	2.65
Spin filter sparging + perfusion	92.0	15.00
Oxygen (10 ml l ⁻¹ min ⁻¹ , 80 r.p.m.)	51.0	8.50
Spin filter sparging	92.0	15.00
Spin filter sparging + perfusion	92.0	15.00

* Assuming oxygen utilization rate of 2.6 µg/million cells/hr.

oxygenator and back into the culture, but for success a fast perfusion rate is needed. The oxygenator can be a capillary fibre device, silicone tubing (37), or a specially adapted reservoir vessel (26). The removal of cell-free medium has been achieved using both gravitational settling methods (9) and spin filters (35). The critical factor is keeping the cells from blocking the filter used for removing the medium. In microcarrier culture, where a mesh size of 60–100 µm can be used, this is relatively straightforward and perfusion rates of at least 2 volumes hr⁻¹ can be achieved. It is more difficult for suspension cultures, as a filter mesh of under 10 µm is required and this only allows perfusion rates of 1–2 volumes day⁻¹ without blockage occurring. The importance of perfusion in culture oxygenation is exemplified by the data in Table VI (32).

Two main methods have been employed to overcome the difficulties of sparging and the complexities of perfusion, apart from increasing the partial pressure of oxygen or the atmospheric pressure of the culture. These are aerating the medium in a cell-free compartment within the culture by means of a mesh screen (33, 35, 88, 105), or by using a bubble-free system based on diffusion through thin vinyl or polypropylene tubing (18, 47, 66). This latter method originally used tubing arranged around the vessel periphery, but owing to the great lengths involved it was rather inconvenient to use, although commercial models are available (55). A more practical means of organizing the tubing is by using the stirrer as a holder (membrane agitator) (39)—this allows both an efficient presentation of the tubing and a large-surface-area impeller. The disadvantages of this aeration method are that scale-up is linear (i.e. tubing length has to increase disproportionately during volume scale-up) and different gases vary in their diffusion rates so that gases cannot be mixed

(e.g. oxygen and carbon dioxide). However, yields of 8×10^6 cells ml^{-1} on Cytoflex microcarriers have been reported (59).

Oxygenation of cultures is a complex subject. If gas bubbles are used, then a high aspect ratio increases the efficiency owing to the higher pressure within the culture and the residence time of the bubbles—this is needed to offset the disadvantages of using large bubbles. If bubbling is not used, then a very low aspect ratio has to be used to increase surface aeration. Oxygen is toxic at concentrations above 21% partial pressure and care has to be taken if this concentration is increased in the gas mixture—in fact, it should only be used in efficiently mixed systems, and well into the culture cycle when the oxygen demand is high. Fortunately, cells seem to find a low oxygen concentration (about 10% partial pressure) optimal. The problem of foam has to be resolved in sparged cultures, especially when serum is present. Antifoam agents, such as silicone antifoam (Sigma, 6–30 p.p.m.) are effective, but should not be used over long culture periods as they do have detrimental effects on cells.

2.4. Perfusion

Perfusion is a long-established technique in cell culture (since 1912). Initially it was used to keep cells viable in very small chambers used for microscopical examination. However, the realization that cells are better suited to a continuous supply of nutrients and removal of toxic waste products than to the alternative fasting and fasting routines of a batch culture, has led to a widespread use of this technique. Small-scale applications probably culminated in the dual-rotary-circumfusion system (78), which allowed the progression and maintenance of differentiated foetal tissue to be microscopically examined in 24 replicate chambers. Medium replacement was carried out by the automatic gravitational filling and emptying of chambers as they revolved on a wheel, and not by pump action as used in subsequent systems.

The current use of perfusion is in response to the need to maintain high cell densities in a unit volume (or unit surface area). This technology originated in 1957 with the Cytogenerator (Fig. 3), a U-shaped tube with its side-arms constructed of sintered glass surrounded by the medium reservoir (23, 24). This development was of interest because (a) it allowed high-density growth of suspension cells (an unheard of $1-2 \times 10^7$ cells ml^{-1} in 1957) and (b) it had a very gentle agitation/mixing mechanism based on the flow of perfused medium and a tidal action created by alternating pulses of air to the tops of the U-tubes. The inevitable problem of filter blockage was delayed by reversing the direction of medium flow every 24 hr. The volume of the reactor, and the reservoir, was 300 ml. At a perfusion rate of 600 ml day^{-1} a density of 1×10^7

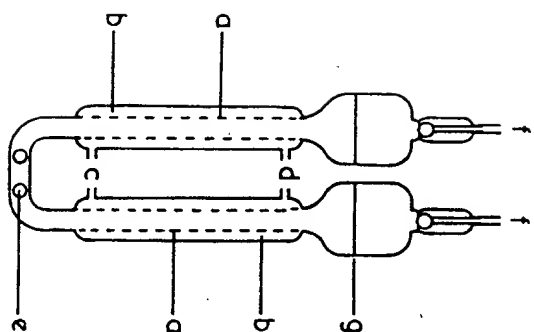


Fig. 3. Cytogenerator (21). A, sinter glass tube; B, outer medium reservoir; C, medium inlet from reservoir; D, medium outlet; E, sampling port; F, gas inlet; G, medium level when stationary.

BHK cells ml^{-1} was maintained for 1000 hr with 150 ml being withdrawn from the culture every 24 hr (25). The efficiency of the filter did decrease after an average of 650 hr. Unfortunately, the U-shaped configuration made the apparatus difficult to scale-up, but it did establish many useful principles and it is surprising that more use has not been made of this (for 1957) advanced technology.

The success of the Cytogenerator for suspension cells provided the impetus to develop perfusion systems for substrate-attached cells. The first of these was the glass helix perfusion chamber (64), of 40 ml capacity and used at an initial perfusion rate of 2 volumes day^{-1} but increasing to 400 volumes day^{-1} in the later stages of the culture cycle. Cell yields in the order of 10^6 ml^{-1} were obtained.

This technique has been developed further by many laboratories using glass pipes (37) and spheres (7, 26, 77, 106, 107) at scales up to at least 100 litres (104). In the 1960s, large-scale production of attached cells could only be achieved with multiple-batch roller bottle culture. By means of specially designed swivel caps, Kruse (51) was able to transfer to roller bottles his

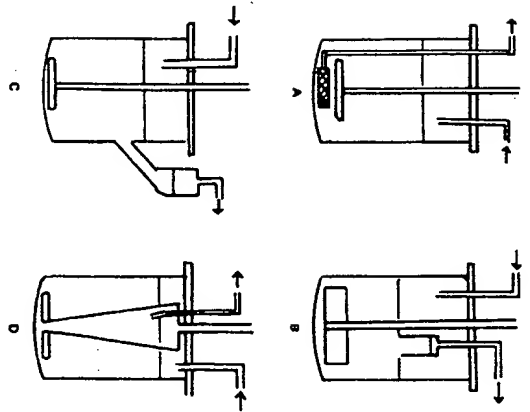


Fig. 4. Devices to separate cells from medium for perfusion culture. A, stainless steel mesh filter (98); B, gravitational settling (internal) (9); C, gravitational settling (external) (16, 47); D, centrifugal separation in stirred tank (82).

successful flask perfusion technique (52, 53), in which cell densities of up to 17×10^6 cells cm^{-2} with perfusion rates in the order of $10 \text{ volume day}^{-1}$. This development is commercially available (118), as are modifications which give larger surface areas (by packing the roller bottle with spaced glass tubing) (11, 21, 81). One modification rotates the bottle alternately clockwise and anti-clockwise through 360° to avoid the use of swivel caps (112). Many other developments to increase the surface area per unit volume have been made which rely on perfusion, and are described in Section 2.5.

Perfused suspension (and microcarrier) cultures continued to be developed (Fig. 4) using gravitational settling systems (9, 16, 47, 96), which permit only slow perfusion rates but do not become blocked, or by using the centrifugal force of an enlarged and hollow stirred shaft (82), or by static filters (98). However, most current procedures are based on spin-filter devices or on filtration systems.

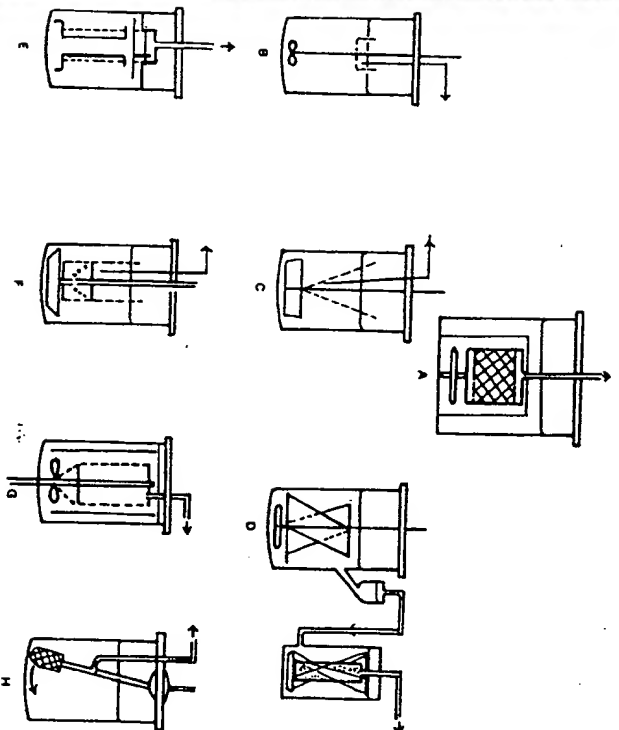


Fig. 5. Development of spin filters. A, Himmelstahl (38); B, wire basket (100); C, internal wire filter (27); D, external filter with spinning tails (16); E, celligen (77); F, spin exchanger (33); G, rotating wire cage (103); H, perfusion wand (111).

2.4.1. Spin-Filter Devices (Fig. 5)

All static filters within a culture become blocked sooner rather than later. This has always been a limiting factor in process intensity scale-up. There are two means of preventing (or delaying) this; they are to provide a large filter surface area (to reduce the flow rate per unit area), or to rotate the filter, thus creating a boundary effect and preventing cell attachment and filter clogging. This principle was first used by Himmelstahl in 1969 (38) and allowed cell densities of $6.5 \times 10^7 \text{ ml}^{-1}$ (L1210 mouse leukaemia cells) to be attained. The filter was a $3 \mu\text{m}$ porosity stainless-steel mesh rotated at 300 r.p.m. and used at volumes up to 1 litre. In later developments (94), microfibre filter tubes, ceramic, and fused glass bead filters were used. This technology was borrowed successfully for the growth of cells on microcarriers where the porosity is not

so critical (27, 28, 34, 100, 101). The same units, made of stainless steel are also currently being used in many laboratories for the growth of hybridoma cells at intermediate cell densities ($1-5 \times 10^7 \text{ ml}^{-1}$) (17, 76, 102). Porcelain filters of $1 \mu\text{m}$ porosity have also been used (95). The limiting factor is that perfusion rates of only $1-2 \text{ volumes day}^{-1}$ can be achieved with small-pore ($5-10 \mu\text{m}$) filters. However, a recent paper (103) describes the use of a filter with a $53 \mu\text{m}$ pore mesh which, by means of a suitably integrated design of fermenter geometry, impeller and cage construction, and stirring speed, allows a 95% retention of suspension cells. The cage is fitted into a draft tube and a perfusion rate of $0.12 \text{ volumes hr}^{-1}$ is used at scales up to 15 litres. Another modification of this technique is to have rotating Teflon sails around the stationary filter in a satellite vessel (17, 96). The boundary effect of the sails on a $1 \mu\text{m}$ porcelain filter allowed sufficient perfusion rates to support Walker Rat carcinosarcoma cells at $3 \times 10^7 \text{ ml}^{-1}$. The use of twin drives, one for stirring and the other for a high-speed spin filter, is another possible solution.

Spin filters provide a simple technical solution to scaling-up process intensity within the well-established stirred fermenters. They do, however, limit process intensity (especially for suspension cells) and can also bring to a process a certain degree of unpredictability as to when filter clogging begins to adversely affect, or cause a shutdown, of the culture.

2.4.2. Filtration Systems (Fig. 6)

Various cultures now exist that rely on the use of a range of filtration materials that allow the passage of low-molecular-weight compounds but retain the cells and even, in some examples, the cell product. These methods were pioneered by Gori (22), who developed the dialysis fermenter as a simpler, and more easily scaled-up system, to the cytogenerator. Dialysis membrane was chosen because other filter materials (e.g. sintered glass) encouraged cell growth on their surfaces and this led to severe blockage problems. The apparatus was used as a chemostat with the objective of maintaining maximum growth rates for long periods of time (over 40 days) rather than supporting high cell densities. However, a modern equivalent is available (the Bioengineering Membrane Laboratory fermenter) (113), which has a Cuprophane dialysis membrane (MW cutoff 10 000 daltons) forming an inner chamber. The reactor tank has two independent stirrer shafts, one inside the membrane and the other in the exterior reaction chamber. The unit was designed principally for microbial use, but can be adapted in a variety of ways for animal cells.

Hollow fibres are available in both ultrafiltration and filtration grades and, in conjunction with conventional stirred reactors, can be utilized for perfusion. Two approaches, illustrated in Fig. 6, have been used. In the first, the cells, medium and product are pumped through an external loop to the fibre

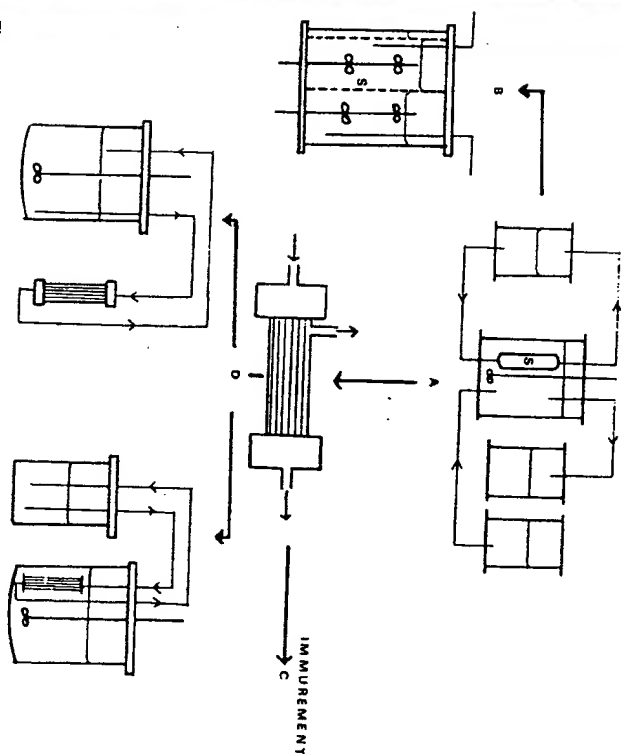


Fig. 6. Culture systems based on filtration membrane principles. A, dialysis fermenter of Gori (22); B, membrane laboratory fermenter (113); C, immersion techniques summarized in Fig. 7. D, hollow-fibre units used in an external loop (109) or internal filter (113); S, dialysis chamber.

cartridge and the cells are returned to the reactor (109). The second method is to perfuse medium through fibres situated in the reactor, i.e. an alternative to spin filters (13). The external loop method has the advantage that the product can be continuously harvested in a concentrated form and the cell density increases within the reactor as they are returned. The disadvantages are potential damage to cells as they are pumped during the loop and the loss of environmental control in the vinyl tubing around the loop and the loss of the loop. The internal system consists of a fibre bundle (MW 6000) only with the cartridge removed and has a greater potential for maintaining high cell densities owing to the ability to rapidly circulate fresh medium. The product has to be harvested in a more dilute concentration with the cells and medium and is thus a semi-batch operation. Both systems are capable of supporting $1-5 \times 10^7 \text{ cells ml}^{-1}$ in the bioreactor and can be a simpler design solution than many other reactor configurations, including the spin filter.

2.4.3. Continuous-Flow (Chemostat) Culture

This methodology was comprehensively reviewed in a previous volume (97) and will not be described in detail here. In the context of a production process, it is only useful for growing (dividing) cells and thus has a limited application unless used as a two-step process. An example is monoclonal antibody production from hybridomas which is not growth-associated. It is a method which provides invaluable research and development data on growth and production kinetics, but has not found a ready application for the manufacture of cell products. One reason is that it is a low-density system providing a continuous trickle of cells and a low-titre product which is far from ideal for downstream processing. Batch culture is a popular concept as a manufacturing process because each batch is a definable entity for quality assurance purposes, initiating from a characterized seed lot. The developmental logic from batch is to a fed-batch, recirculation or perfusion system which allows higher cell densities and product concentration. Continuous-flow culture is only suitable for suspension cells in which the product is growth-dependent, it is low-yielding, and needs an extra downstream processing step to remove the cells. Also, continuous cell division could result in changes to the biological properties of cells and there are always difficulties with animal cells in precisely defining the growth-limiting factor.

2.5. Cell Immobilization

Density scale-up using spin filters, etc., is technically limited to a 10-50-fold increase over non-perfused cultures. To achieve higher cell densities, various immobilization techniques have been developed in order to achieve more efficient and critical control of the cell environment by means of medium perfusion. Basically, two concepts are being used. Firstly, immurement, or the retention of cells within a compartment which allows free passage of medium. Secondly, the entrapment approach, which provides a substrate with the physical configuration to capture and trap cells. Reliance is also placed upon suspension cells weakly attaching to or adsorbing onto the surface of the entrapment matrix. Anchorage-dependent cells benefit from the greatly increased surface area of such materials. Many immobilization materials protect cells against mechanical stresses caused by stirring and high perfusion rates. Although primarily aimed at providing independent high-density systems, they can, in addition, improve the efficiency of spin filter techniques as the support material is so much larger than individual cells.

7. Cell Culture Systems and their Scale-up

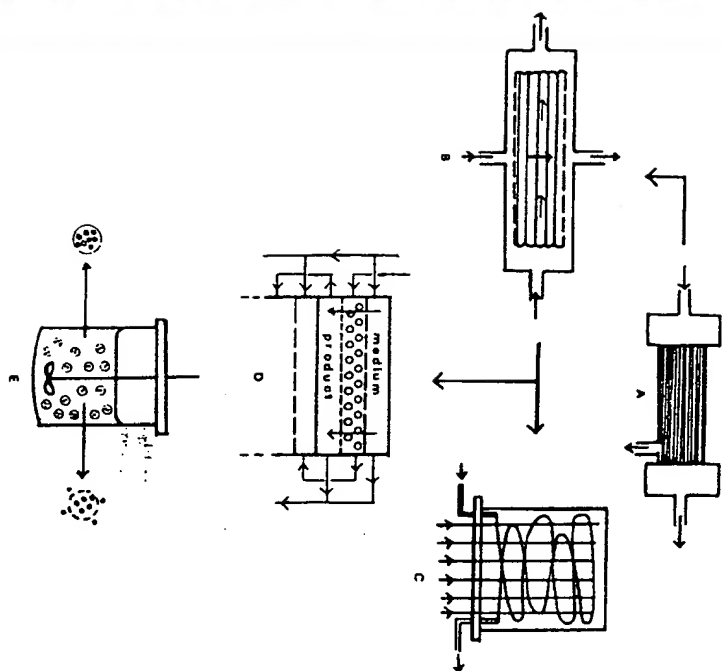


Fig. 7. Cell immurement techniques. A, hollow-fibre reactor; B, flat-bed hollow-fibre reactor (44); C, static maintenance reactor (55); D, membrane reactor (46); E, encapsulation (74).

2.5.1. Immurement Techniques (Fig. 7)

Modified Filtration Techniques. An efficient system for perfusion of high cell densities was developed by Knazek (49) in 1972 using ultrafiltration capillary fibres (cellulose acetate for medium diffusion and silicone polycarbonate for gas diffusion). These original cultures (Amicon Vitabder) were available in 25, 250, and 2500 cm² sizes with extracapillary volumes of 2.5, 25, and 250 ml respectively. The fibres had a spongy wall (50-70 μ m thick) and a central lumen of 200 μ m diameter. The external surface was very porous, but the lumen lining was a thin ultrafiltration skin with either 1000, 5000, or

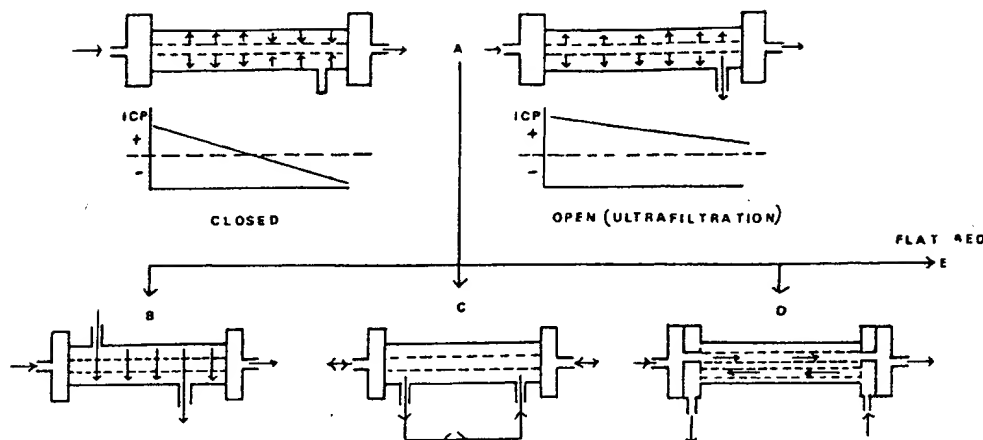


Fig. 8. Homogeneity and scale-up problems of hollow-fibre reactors, and possible solutions. A, flow path patterns in open (ultrafiltration) and closed systems and the pressure differential between inside (ICP) and outside the fibres which results in uneven growth (91); B, crossflow diffusion of nutrients, with gas flow through the fibres; C, cyclical pressure pulses between the inside and outside of the fibres (115); D, contraflow fibres of nutrients and oxygen; E, flat-bed reactor (illustrated in Fig. 7), an example of crossflow diffusion.

10 000 MW cut-offs. A unit consisted of thousands of fibres "potted" at either end in a cylindrical housing and was capable of supporting $1-10 \times 10^6$ cells cm^{-2} (10^4 cells ml^{-1}). This concept has been developed over the years and is now very widely used in many commercial applications (see Chapters 15 and 16), especially for producing monoclonal antibodies from hybridoma (suspension) cells. There is no questioning the efficiency of these capillary fibre cultures, in which over 10^4 cells ml^{-1} can be maintained for long periods (months) within the extracapillary compartment. However, there is a scale-up problem in that pressure and concentration gradients build up along the length of the cartridge (Fig. 8). This phenomenon has been explained in detail (91), and is due to transmembrane flux being directed back into the lumen for the latter half of the flowpath in the closed (re-cycle) mode (i.e. when medium passes through the lumen only). This results in much higher cell densities at the inlet end compared to the outlet end, with suspension cells, a packed cell mass accumulating at the outlet end. In the ultrafiltration mode (if the extracapillary volume is continuously being withdrawn) the pressure drops continuously along the length of the cartridge, again resulting in uneven growth. Both configurations are therefore severely limited for scaling-up. This particularly applies to reactors using ultrafiltration fibres, chosen because of their ability to compartmentalize the cell and product away from the bulk of the re-circulating medium. Some systems are based on filtration-grade membranes ($0.1-0.22 \mu\text{m}$) in order to achieve a greater flux rate (10, 92), i.e. only the cells not the product are retained in the extracapillary space. Scale-up is mainly limited to providing a series of replicate units, although attempts to fabricate larger units are being made. Methods of overcoming this gradient problem include the insertion of contraflow and aeration fibres (92), a cyclical flushing of medium either from the lumen into the extracapillary space or into the lumen from the extracapillary space (115), and the use of flat-bed reactors (96, 16, 54) rather than the cylindrical filtration cartridges (Fig. 8).

Tharakan and Chau (92, 93) have designed a unit that combines the high surface-area-to-volume ratio of hollow fibres with the efficient feed delivery of a radial flow reactor. This culture consists of a central perforated stainless-steel radial flow distributor, which gives a uniform delivery of nutrients along the total length of the fibres, and an annular bed of hollow fibres through which the gas mixture flows. Medium diffuses from these fibres and is carried away by the gas flow. This arrangement permits considerable scale-up whilst maintaining homogeneous conditions.

The concept of using pressure differentials to simulate *in vivo* arterial and venous flow is used in the Acusyst (115) system. The culture has a dual medium circuit, one passing through the lumen of the fibres, the other through the extracapillary space. By cyclically alternating the pressure

between the two circuits, media is made to pass either into or out of the lumen. This allows a flushing of media through the cell compartment and overcomes to some extent the gradient problem. It also allows extra concentration of the product just before harvesting. The system is described fully in Chapter 16.

Another means of overcoming the concentration gradient limitation to scale-up is to use a flat-bed hollow fibre perfusion reactor (16). The fibres are used for aeration, and as an attachment surface for the cells; 3–6 layers of fibres are sandwiched between steel microphore filter plates (20 μm pores), between which the medium is perfused. Densities of 10^6 cells cm^{-2} have been maintained for periods of 60 days. The flat-bed configuration brought about a 4-fold increase in cell yield over the cartridge-type owing to the short nutrient flow path preventing any gradients or the need to use fast shearing flow rates. It provides a very good basis for scale-up and in fact it has allowed the development of the In Vitro Static Maintenance Reactor (SMR) (Chapter 17) which uses silicone tubing for gas exchange and fibres for nutrients (96). The spacing of the fibres (5 mm) seems somewhat excessive for optimum conditions, but the reactor is for cell maintenance only—cells are grown to high densities in other cultures (either suspension or microcarrier), and mixed with the matrix material in the SMR. A similar concept, but using just the membrane sandwich without fibres, is the Membroform Bioreactor (48) (see Chapter 11). In this system, membranes of different porosities can be used, so that one can have consecutive arrangements or combinations of medium, cell, and product compartments.

Hollow-fibre culture has mainly been used for suspension cells, particularly hybridomas, with fibres based on cellulose acetate. It is now becoming increasingly possible to grow anchorage-dependent cells in the same system using fibres based on polypropylene. A 1-litre cartridge, which supports over 10^{10} suspension cells, has a surface area of approximately 1 m^2 . The normal expectation is a yield of $1\text{--}2 \times 10^5$ cells cm^{-2} , thus a cartridge would be expected to support $1\text{--}2 \times 10^8$ cells but it is claimed that the yield would be higher owing to the process control facilities of this type of equipment. Quantitative data on cell yields are difficult to determine with any degree of accuracy in this type of system, but figures of $1\text{--}2 \times 10^6$ cm^{-2} have been confirmed (54).

There are several means of overcoming the problem of poor attachment to fibres. One solution is to coat polysulfone 351000 fibres (Amicon) with poly-D-lysine which results in yields of over 10^7 fibroblast cells per cm^2 (92). Another approach is to pack the extracapillary space with microcarriers (90). Both polyacrylamide and dextran microcarriers have been successfully used.

Besides the scale-up problem, a serious disadvantage of filtration fibres is that many types cannot be steam-sterilized and ethylene oxide sterilization has to be used followed by careful aseptic assembly of the whole unit.

Polysulfone and Teflon fibres can be steam-sterilized. Also, many of the fibres have toxic components which have to be leached out before cells are added.

To summarize, hollow-fibre systems have large surface-area-to-volume ratios, continuous removal of waste products, high cell densities which allow a tissue architecture to be established, separation of the cells from the nutrient flow, thus eliminating the effects of shear, etc., and the possibility of concentrating the product in a small volume. Disadvantages are diffusional limitations, especially for homogeneity and scale-up, process control complexity, sterilization method, possibility of fibre blockage and membrane leakage, and sampling difficulties.

Microencapsulation. A totally different approach, but using similar principles, is the microencapsulation technique, originally developed by Mosbach and co-workers (70) and Lin and Sun (60), and currently being used for the commercial production of monoclonal antibodies (12, 80). There are basically three different methodologies currently in use. The first, Encapcel (12, 80) (Damon Biotechnology), traps the cells in sodium alginate spheres, which are then coated with polylysine to form a semi-permeable membrane. The internal gel is then solubilized with sodium citrate, which releases the cells into free suspension within the capsule. Cells grow within the sphere from a seed of 2×10^6 to a final intracapsular concentration of 500×10^6 ml^{-1} after several weeks. The second method, Geltrap (Karyon technology), is a simplified version of the preceding method using calcium alginate only, which allows products such as antibody to diffuse into the culture medium. A problem with this method is that spheres tend to be rather large (0.5–1 mm), which causes severe nutrient limitation in the centre. The third method uses agarose beads in which the cells are within a honeycombed matrix within the gel (71, 73). The agarose fragments, unfortunately, have a wide size distribution and a lower mechanical strength compared to alginate (74). Other materials that have been used are collagen and fibrin, mainly to promote encapsulation systems for anchorage-dependent cells.

The advantages of these techniques are that the cells and (with a suitable capsule membrane such as the Encapcel) product are compartmentalized into small (500 μm) capsules which can be stirred in conventional fermenter equipment. Thus volumetric scale-up possibilities are far greater with this technology than with the fibre systems. The fragile cells are protected from mechanical stresses (thus higher stirring speeds can be used) and can be kept in serum-free medium (serum is in the extracapillary medium). In the Encapcel method cell densities of over 10^8 ml^{-1} are achieved and a high product concentration accumulates within the capsule owing to the molecular cut-off of 100 000. However, the system is restricted to a batch operation, as at the end of the culture period (10–15 days) the capsules are allowed to settle out and the

concentrate of capsules is lysed to release the product. To date, the method appears to be restricted to below the 100-litre scale and cell viability within the capsule is difficult to maintain. It is also a highly sophisticated and complicated procedure, and difficulties will be met with products that cause negative feedback problems to the cell. The other encapsulation technologies do offer the prospect of longer culture duration, as the product can diffuse out into the bulk culture fluid, and also since agarose is more stable than many other polysaccharide gels in culture media. However, the method is still considered at best a pilot-scale technology and not suitable for industrial scale-up, presumably because of the logistical and technical complexities.

A recent commercial development is the Belco Bioreactor (14) which is a conical design and relies on airlift to circulate cells in, or on, dense slurries of hydrogel (alginate) beads. The beads are generated within the reactor and the 3.5-litre working volume is claimed to have 200 cm² ml⁻¹ surface area.

Product Compartmentalization. The ability of some of these various immured culture systems to allow a product to become concentrated within a small compartment of the total medium volume is summarized in Table VII. Between 1.2% and 2% of the total volume (culture plus reservoir) can be isolated for product accumulation and this can be very advantageous for downstream processing.

TABLE VII Compartmentalization of Cells and Product Within the Bulk Medium

Method	Ratio		
	Product volume	Reactor medium volume	Total medium volume
Encapsulation	1	3	80
Membroform	1	3	50
Hollow fibres	1	2	80

2.5.2. Entrapment (Fig. 9)

A methodology that is being increasingly used is entrapment. Inertial entrapment systems allow a fluid flow over a textured, or partitioned, surface which traps the cells and prevents them being washed through the unit. Examples are the tubular flow reactor (46) (Chapter 9) and the Optical System (4) using the S-Opticore cartridges (Chapter 14). The claim is that over 95% of the cells are retained in the ceramic surface architecture, but some reliance is placed on the suspension cells "weakly" attaching to the surface. A

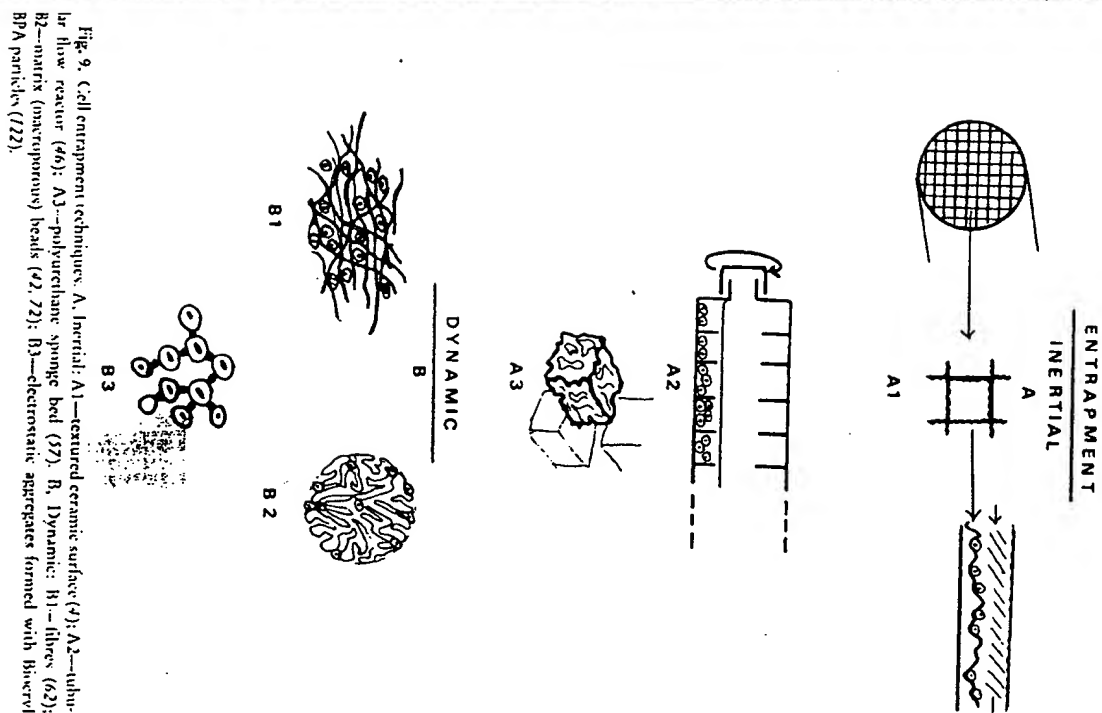


Fig. 9. Cell entrapment techniques. A, Inertial: A1—textured ceramic surface (4); A2—tubular flow reactor (46); A3—polyurethane sponge bed (37). B, Dynamic: B1—fibres (62); B2—matrix (macroreticular) beads (42, 72); B3—electrostatic aggregates formed with fibrillar BPA particles (122).

recent development is the use of polyurethane sponges, in the form of 0.5-1 cm³ cubes, in a packed column (37). The medium is free to circulate around the cubes and the porous matrix of the sponge entraps the cells as the medium is continuously circulated. Dynamic entrapment methods are also available based on clumps of (cellulose) fibres which entmesh cells in a stirred culture (56, 62) or a matrix-type microcarrier bead (42), or simply entrap them in a gel sphere (72).

An advantage of this type of culture is that volumetric scale-up possibilities are far greater than with immured systems, because of the lower pressure gradients across the bed. Thus, faster perfusion rates, with less sophistication of design, can be used which allow far larger volumes to be used before nutrient concentration gradients limit the scale-up. In addition, most of the substrates can be steam-sterilized.

2.6. Cell Substrates

Some cells need to be attached to a suitable surface either because they cannot grow in free suspension (anchorage-dependent cells, e.g. human diploid fibroblasts), or because expression of cell products are significantly lower, or altered (85), when cells are grown in suspension culture. Despite the unchallenged fact that cell production is more economical, and scale-up is more efficient, in suspension culture, substrate-attached cells still have a very important role in cell biotechnology.

Traditionally, scale-up of substrate-attached cells has been to increase the number of small culture units (flasks, roller bottles, etc.). This procedure is not only wasteful in labour but requires large volumes of working and incubation space. In addition, critical environmental control procedures which improve the productivity and reproducibility of cultures cannot be employed. The aim, therefore, is to scale-up by changing from a multiple to a unit process. This requires the provision of a large surface area in a compact volume in which media can flow through at a rate sufficient to keep the cells at optimum performance levels. A useful measure of the relative efficiency of various culture reactors in this respect is to compare them in terms of ratio of surface area to reactor medium volume. This parameter has been used to summarize anchorage-dependent culture systems in Fig. 10. Many of these methods have already been reviewed conceptually (Volume 1, Chapters 7-11) and only those currently being developed, or used commercially, are considered here. Basically, scale-up methods are based on either plate, immobilized beds, fluidized beds or membrane (cartridge) concepts.

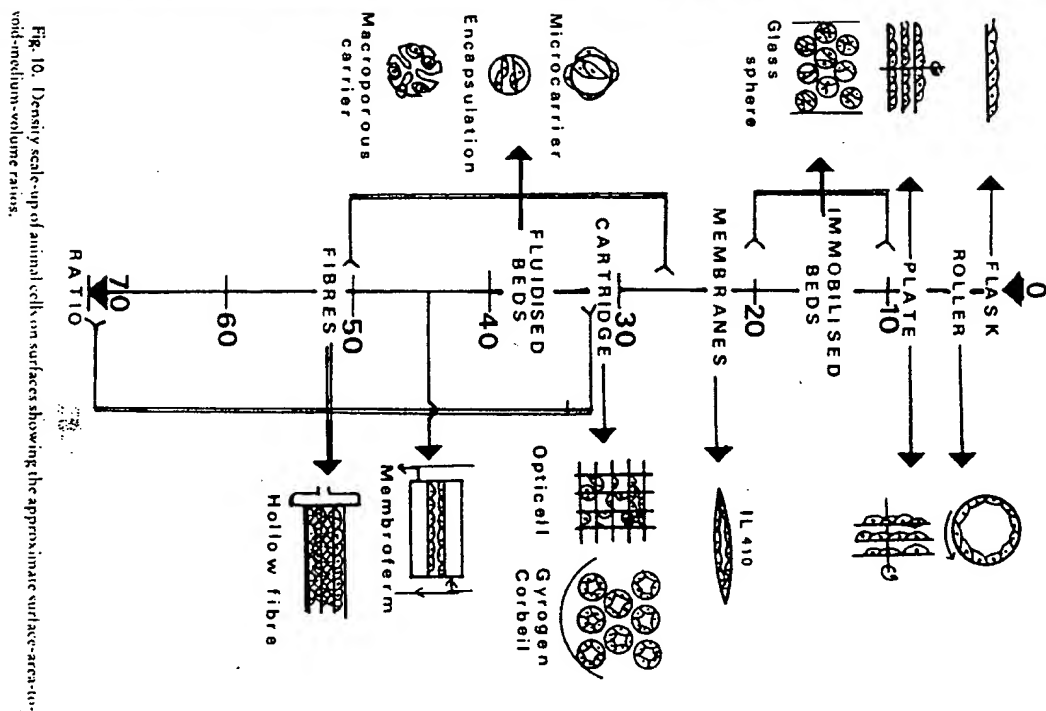


Fig. 10. Density scale-up of animal cells on surfaces showing the approximate surface-area-to-volume ratios.

2.6.1. Plate Reactors

The inclusion of stacks of parallel plates in either a horizontal or vertical mode is a simplistic way of increasing the surface area within a reactor. Many systems have been developed and used at scales up to 200–300 litres (67, 83). However, they all have serious limitations. Vertical plates are difficult to inoculate evenly and, when confluent, the cell sheets have a tendency to slide off. Horizontal plates need a tilting mechanism to remove medium from between plates, only half the surface area can be used for cell growth, and there can be no variation in the surface-area-to-medium-volume ratio. However, high cell densities ($1 \times 10^6 \text{ cm}^{-2}$) have been obtained in glass plate units of 10 m^2 (63). This particular system has been used for the manufacture of measles vaccine.

One type of plate reactor which was thought to overcome many of these problems is the plate heat exchanger (Fig. 11) (7, 8, 33, 35, 110). This apparatus consists of a frame in which independent metal plates, supported on rails, are clamped between a head and follower. The stainless-steel plates (type 316) are sealed at their outer edges and around the ports by gaskets which are arranged so that the process fluid and the warming fluid are directed alternately into the passages formed by the plate. Although the plates are vertically stacked, they are ribbed in a herring-bone pattern, which means that most of the surface area is at 45° . However, cell attachment and growth in this apparatus is uneven except for cells which have a very rapid attachment time, or fibroblasts which are highly motile and can thus compensate for attachment gradients; i.e. the system is more suited to fibroblast than epithelial cells. Such cells grow to densities compatible with other substrates ($8\text{--}10 \times 10^4 \text{ cm}^{-2}$). Nevertheless, the linear scale-up capacity of the system is enormous, as a rig can hold 100 plates available in surface areas from 850 cm^2 to 3.25 m^2 . With suitable modifications it has the potential to be an efficient large-scale, although only low-intensity, culture unit.

2.6.2. Immobilized Beds

Many different substrates have been used to form an immobilized bed suitable for animal cells (for review see ref. (87)). The most-used substrate is the glass sphere, despite the fact that a sphere has the lowest surface area to volume ratio possible. An immobilized bed matrix has to be a compromise between maximizing the surface area and providing a passageway open enough for medium to be perfused at a rate sufficient to maintain homogeneity throughout the bed without subjecting the cells to damaging shear effects. Recent data (63) have demonstrated that a pore size of over 5 mm diameter is needed for maximum growth. The data in Table VIII show that using differently sized glass spheres in the diameter range 2–8 mm, the total cell yield

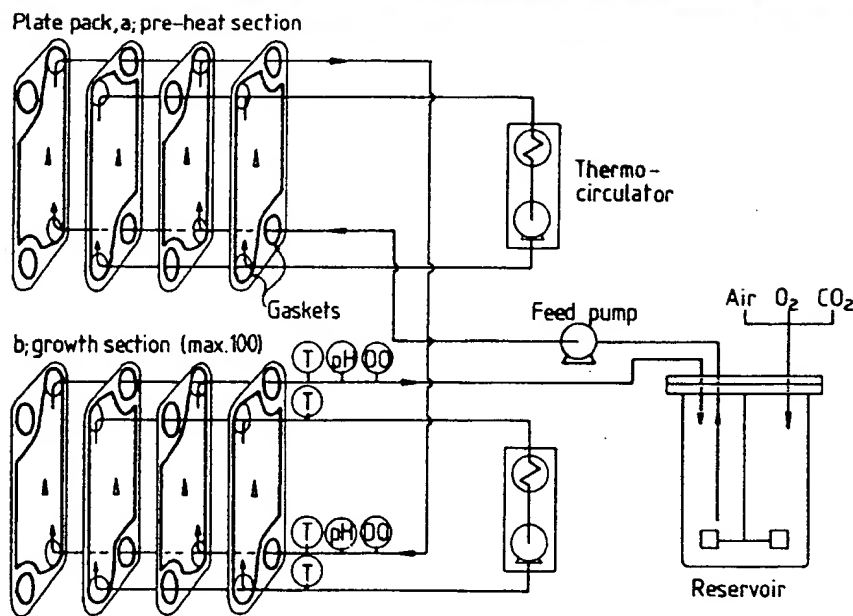


Fig. 11. Plate heat exchanger (110). Diagrammatic representation of individual plates in the culture and a flow diagram of the culture in operation (T, temperature sensors; pH, DO, pH and oxygen sensors).

TABLE VIII Effect of Glass Sphere Diameter on Cell Yields

Bead diameter (mm)	Cell number per		
	10 ³ cm ²	10 ³ kg beads	10 ³ ml
2	0.36	6.7	2.7
3	0.78	4.0	1.6
4	1.25	5.0	2.0
5	2.50	8.0	3.0
6	2.20	6.0	2.4
7	2.40	5.0	2.0
8	2.30	4.3	1.7

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was higher in beds of the larger spheres despite the reduction in total surface area. The interpretation is that the larger channel size overcame the problems of inhomogeneity and channelling (preferential flow through selected channels). To reduce inhomogeneity problems, the glass spheres could be coated with Fibronectin or the direction of medium flow could be alternated every 24 hr. In large beds, cells should be inoculated through a perforated tube running centrally the height of the bed to obtain an even distribution of cells (63).

Immobilized-bed cultures based on glass spheres have many advantages; in that the substrate is both inexpensive and re-usable, and scale-up to at least 100 litres is relatively problem-free (104). The disadvantages are that it is not a high-intensity system and can only support low cell densities per unit area; and it is limited to products that are secreted, i.e. it is ideal for long-term continuous cultures (see Chapter 10) but not for batch or cultures in which cells have to be harvested or the product extracted.

Several commercial systems based on packed cylinders of glass tubing have been developed. The simplest is the Corbiel which is used as a high-surface-area roller bottle (15 000 cm²) for the manufacture of vaccines (11). The Gyrogen monolayer tissue culture fermenter (Chemap) was also developed for vaccine (FMDV) manufacture but is far more complex, with full process control capability (21). The largest unit provided 34 m² of surface area, but the system never became popular, mainly because it was complicated to use and to standardize, was not very versatile, and was expensive for the degree of process intensity and control sophistication it offered.

2.6.3. Fluidized Beds

Microcarrier Culture. Microcarrier culture, which can be considered as a dilute form of fluidized bed, is one of the few systems that has gained some

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acceptance as a commercially viable scale-up culture process. It is being used by several companies at scales exceeding 1000 litres for vaccine manufacture (66). The method has been greatly refined since van Wezel (99) published his preliminary results in 1967. Problems of unsuitable surfaces for attachment, even carrier toxicity, have been overcome and currently there are over 20 types of microcarrier commercially available (see Chapter 12). The advantage of being able to monitor and control the environment and achieve true homogeneity has allowed considerable scale-up in both volume and process intensity (to 5×10^7 cells ml⁻¹). Cell density has been increased by using high concentrations of microcarrier (e.g. 15 g l⁻¹) and maintaining the correct environment by medium perfusion. This has largely been made possible by the development of efficient spin filter systems for aeration (88, 105), and perfusion (101, 28), or both (32, 34, 35, 117). The ability to use fast perfusion rates (up to 2 volumes hr⁻¹) is extremely important for both nutrient supply and oxygenation (Table VI) (32).

Improvements in the mixing efficiency of specially designed flat-blade impellers which allow slow stirring rates to be used, and are thus potentially less damaging to cells, means that the possibility of microcarrier aggregation has to be monitored. This aggregation is usually a deleterious event, and the stirring speed should be increased above that necessary to maintain homogeneity in order to prevent it occurring.

Many efforts have been made to effect cell transfer from one bead to another within the culture (19, 39, 61). This is to facilitate the progressive scale-up of the cell seed. However, success is very limited and usually results in a very uneven distribution of cells between the beads. Although more costly, a good means of preparing a large cell inoculum in excellent physiological condition is to grow seed cultures on gelatin microcarriers. These are very easy and quick to dissolve, and leave the cells completely undamaged. This can even be done within the same reactor vessel in which the next higher volume of culture is to be grown. It also solves the perennial scale-up problem of how to prepare a large cell inoculum (e.g. for a 100-litre culture) quickly before trypsinization damage occurs.

The flexible process capabilities of microcarrier culture is exemplified in Fig. 12 (34). This is a 100-litre volume culture vessel with a spin filter that allows a microcarrier (Gyrodex 3) concentration of 12 g l⁻¹ to be used. Cells are inoculated when the medium level is low (L2) and when attachment is complete this is raised to 100 litres (L1). The culture is perfused from the 30-litre reservoir vessel (medium at L3 mark), in which all environmental control procedures are carried out, at a rate of 2 volumes hr⁻¹. When the growth phase is complete, the medium levels can be reduced to the L2 and L4 marks (Fig. 12) so that perfusion can continue and a much higher product concentration can be achieved—in effect, the cell concentration is increased

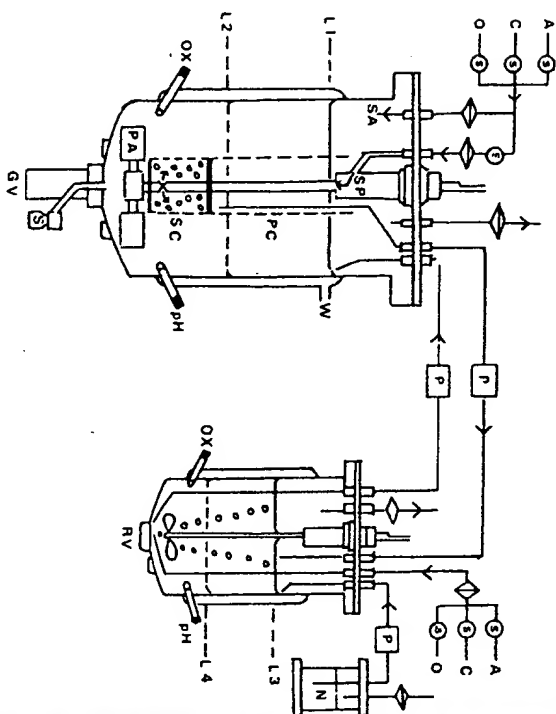


Fig. 12. 100-litre cell culture vessel (GV) with spin exchanger (SA, 117) and a 30-litre reservoir (RV) in a complete perfusion culture system for high-density microcarrier culture. A, air; C, carbon dioxide; O, oxygen; N, NaOH; P, pump; SA, surface aeration; SP, sampling; L2, inoculation level; L1 and L3, growth levels; L4, product manufacture level; S, sampling; Pa, stirrer paddle; W, water jacket.

from approximately 2×10^7 to 4×10^7 ml^{-1} . A complete change of medium (e.g. to serum-free) can also be easily carried out between the growth and production stages by allowing the microcarriers to settle out.

Matrix (Macroporous) Carriers. It can be postulated that a microcarrier concentration of 15 g l^{-1} (27.5% of the total culture volume) may be the upper limit of a stirred system, owing to mechanical factors such as bead collision. There are two ways in which the method can be developed further, namely to provide a "matrix (porous) bead" which relies on protected surfaces within the bulk of the sphere, or to increase carrier density using fluid-lift, rather than stirred, mixing principles.

The use of matrix beads in a true fluidized bed in which 80% of the culture volume is the bead is described in Chapter 13 (42). In this system (CF-1MMO,

Verax Corp.) a density of 4×10^7 cells ml^{-1} of reactor volume, and $2-3 \times 10^8$ cells ml^{-1} inside the reinforced collagen sponge bead matrix, is run on a continuous basis. The microbeads are 200–600 μm in diameter with internal interconnecting pores of 30–100 μm , and are cultured as a thick slurry. Typically, about 6×10^6 beads per litre are used, giving a total surface area of $300 \text{ m}^2 \text{ l}^{-1}$. This system is suitable for both suspension and anchorage-dependent cells and the three-dimensional morphology of the matrix can be altered to meet the requirements of the different cell types (42).

A recent paper (72) has described the fabrication and potential use of macroporous gelatin beads. These gelatin spheres have large cavities and are capable of supporting double the number of similarly sized solid spheres. As well as affording mechanical protection, these cavities also allow the cells to develop and maintain a suitable microenvironment. The potential of fluidized-bed systems is being recognized by the appearance of several commercial reactors (14, 113).

The many natural advantages of microcarriers over other competing systems (Table II), coupled with the developments discussed above, should make this technology one of the dominant commercial systems of the 1990s, coupling as it does volumetric and density scale-up. It is a method of great versatility with a huge range of surface configurations, the ability to be used in a wide range of reactor types from roller culture to airlift (if antifoam is added), in fibre cartridges (90), and in three-dimensional rotary-swelling reactors (114). One disadvantage for some users is the high cost of the substrate if a reusable type is employed. However, the high cost of regenerating a substrate plus the high value of most cell products actually make substrate costs a fairly low proportion of the total costs.

2.6.4. Membrane Systems

Membranes were initially used for the growth of cells because they not only provided a large surface area but were a convenient means of aeraing the culture. Early examples are the polymeric fluorocarbon bags (69) and the LL410 reactor (41) and the concept has now been utilized for the growth of suspension cells in specially developed plastic bags (1 litre) that allow rapid gas exchange (50). Cells preferentially absorb nutrients from their peripheral membranes and from the attachment surface; thus, growth on permeable membranes would seem very advantageous. However, despite these cultures being based on such sound principles, they have not been used very extensively outside their laboratory of origin, possibly because they are over-elaborate in design. Owing to the present need to support high cell densities, membrane systems are now getting far more attention. The main examples, hollow-fibre units (Section 2.4.2) and the Membroform (48), have been mainly used for suspension cells but have subsequently been modified for

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TABLE IX Scale-up Factors—A Comparison of Different Culture Systems

(A) Anchorage-dependent cells								
Factors	Roller bottle	Plate	Immobilized bed	Fluidized bed		Cartridge		Membrane
				Microcarrier	Matrix	Ceramic	Fibre	
Scale-up potential	0	2	2	3	3	2	1	1
Process simplicity	0	2	2	1	1	2	1	1
Mass transfer efficiency	3	1	2	3	2	2	3	3
Aseptic operation	1	3	2	3	3	3	1	1
Direct monitoring	3	0	0	3	2	0	0	0
High cell density	0	0	1	2	3	1	3	3
Steam sterilizability	(Y)	Y	Y	Y	Y	Y	N	Y
Downstream compatibility	0	1	2	2	3	2	3	3
Re-utilizable substrate	(Y)	Y	(Y)	N	N	(Y)	N	N
Flexible area: volume ratio	3	0	1	3	3	1	3	2
Homogeneity/mixing	1	2	2	3	2	1	1	3
Critical control	0	1	2	2	2	2	3	2
Continuous process	0	1	3	3	3	3	3	3

(B) Suspension cells								
Factors	Airlift	Stirred	Spin filter	Membrane	Ceramic	Fibre	Encapsulation	Gel Entrapment
Scale-up potential	3	3	3	2	1	1	1	2
Process simplicity	3	2	2	1	2	1	0	1
Mass transfer efficiency	3	2	3	3	2	3	1	2
Aseptic operation	3	3	3	1	3	1	1	1
Direct monitoring	3	3	3	1	0	0	1	0
High cell density	1	1	2	3	2	3	3	3
Steam sterilizability	Y	Y	Y	(N)	Y	N	(Y)	(Y)
Downstream compatibility	1	1	2	3	2	3	3	1
Homogeneity/mixing	3	3	3	2	2	1	1	2
Critical control	2	2	2	2	2	3	1	2
Continuous process	1	1	2	3	2	3	0	2

Key: 0—not possible; 1–3—increasing ease or efficiency; Y—yes; N—no; ()—with some alternatives.

substrate-attached cells. Of the range of fibre materials available, polystyrene has been found to be most suitable. Attachment efficiency is increased by coating with poly-D-lysine (92).

If the membranes were totally covered by cells, a problem would arise of getting sufficient diffusion of nutrients into the cell compartment; thus in practice a lower-than-average cell yield is to be expected (possibly only 20% of the theoretical maximum). The cyclical flushing process between the lumen and extracapillary space used in one system (115) partially offsets this problem.

Membrane-type culture units have a great theoretical potential since they allow a maximization of surface area with the means of feeding the cells without having to rely on fast, shearing, medium flow rates. In practical terms such culture units only represent small-scale systems (below 1 m²) at the moment, but there is a lot of activity in fabricating units which use various combinations of membranes, fibres and ceramics in order to scale up the size.

3. CONCLUSIONS

There is still a huge scope for improving reactors further to give greater versatility and scale-up capability. With scale-up the potential loss if a culture succumbs to contamination or system failure in terms of labour, materials, and time becomes increasingly costly. Thus, a keyword in design must be simplicity, since complexity of equipment design, media, support systems, and operational manipulations all lead to contamination risks. The parameters one looks for in a large unit process are listed in Table IX. Obviously, no one culture can feature all of these characteristics and a choice has to be made depending upon what is considered most important for a particular process. In order to compare the systems described in this review, each one is scored for each parameter in Table IX. These values should be taken purely as a guide and not as absolute values. There are several reasons for this, including personal preferences or interpretations, and also the fact that modifications which individuals have made (many of which have been described in this chapter) in order to reduce a problem, or to increase performance, have not been allowed for. The data are intended as a quick means of comparing different technologies and allowing a preliminary screening of which one might be the most suitable for a particular application.

Reactors should not, of course, be thought of in isolation, or as the key to improving productivity of animal cells. A process is as strong as its weakest component and therefore supporting equipment should not be ignored: items such as valves, gauges, sampling devices, attachment surfaces, etc. Probably the biggest increases in productivity will come as a result of studying cell

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physiology in more depth, and from media development. However, for this knowledge to be properly used we are largely dependent on the development of reliable and new biosensors (e.g. for glutamine, ammonia, lactate, and glucose). Computer technology is advanced enough to take advantage of such developments to give precise on-line monitoring and process control.

In conclusion, the ideal of a large-volume dense-culture system is tantalizingly close but needs technological developments in several related areas to bring it about. In the following chapters there is a chance not only to select a system which comes nearest to fulfilling one's present requirements, but also to try to decide which of the current culture systems or concepts will become the dominant technology.

REFERENCES

1. Aarhaugen, W. R., and Birch, J. (1986). Large-scale culture in biotechnology. *Science* **232**, 1390-1395.
2. Barclay, S. J. (1984). The skull fermenter. *Dev. Biol. Stand.* **55**, 143-147.
3. Bell, G. H., Davidson, J. N., and Scarborough, H. (1956). "Textbook of Physiology and Biochemistry" (3rd edn), p. 528. E. F. Livingstone Ltd., London.
4. Berg, G. J. (1985). An integrated system for large scale cell culture. *Dev. Biol. Stand.* **60**, 297-307.
5. Birch, J. R., Thompson, P. W., Lambert, K., and Boraston, R. (1983). The large-scale cultivation of hybridoma cells producing monoclonal antibodies. In "Large-scale Mammalian Cell Culture" (J. Feder and W. R. Tolbert, eds.), pp. 1-18. Academic Press, Orlando.
6. Birch, J. R., Thompson, P. W., Boraston, R., Oliver, S., and Lambert, K. (1987). The large-scale production of monoclonal antibodies in airlift fermenters. In "Plant and Animal Cells: Process Possibilities" (C. Webb and F. Mavituna, eds.), pp. 162-171. Ellis Horwood, Chichester.
7. Burdidge, C. (1980). The mass culture of human diploid fibroblasts in packed beds of glass beads. *Dev. Biol. Stand.* **46**, 169-172.
8. Burdidge, C., and Dacey, I. K. (1984). The use of plate heat exchangers in growing human fibroblasts. *Dev. Biol. Stand.* **55**, 255-259.
9. Butler, M., Imamura, T., Thomas, J., and Thilly, W. G. (1983). High yields from microcarrier cultures by medium diffusion. *J. Cell Sci.* **61**, 351-363.
10. van Brunt, J. (1986). Immobilized mammalian cells: the gentle way to productivity. *BioTechnology* **4**, 505-510.
11. Corbett, M., Trundel, M., and Payment, P. (1979). Production of cells and tissues in a new multiple-tube tissue culture propagator. *J. Clin. Microbiol.* **10**, 91-95.
12. Duff, R. G. (1985). Microencapsulation technology: a novel method for monoclonal antibody production. *Trends Biotechnol.* **3**, 167-170.
13. Emery, A. N., Lavery, M., Williams, B., and Handa, A. (1987). Large-scale hybridoma culture. In "Plant and Animal Cells: Process Possibilities" (C. Webb and F. Mavituna, eds.), pp. 137-146. Ellis Horwood, Chichester.
14. Jamiliet, P. C., Smith, C. M., Cullen, B. R., Stremlitz, D. L., and Fredericks, J. D. (1987). Mammalian cell culture production in an airlift bioreactor. *In Vitro* **23**(3), 21A (Abstract 37).
15. Izackes, J. S. (1983). Automated production of monoclonal antibodies in a gyostat. *J. Immunol. Methods* **57**, 121-136.

16. Feder, J., and Tolbert, W. R. (1983). The large-scale cultivation of mammalian cells. *Sci. Am.* 248, 24-31.
17. Feder, J., and Tolbert, W. R. (1985). Mass culture of mammalian cells in perfusion systems. *Int. Biotech. Lab.*, June, 40-53.
18. Fleischaker, R. J., and Sinkov, A. J. (1981). Oxygen demand and supply in cell culture. *Eur. J. Appl. Microbiol. Biotechnol.* 12, 193-197.
19. Gebb, C., Lundgren, B., Clark, J., and Lindberg, U. (1984). Harvesting and subculturing cells growing on denatured-collagen coated microcarriers (Cytodex 3). *Dev. Biol. Stand.* 55, 57-65.
20. Girard, H. C., Okay, G., and Kivileim, Y. (1973). Use of the vibrocentrifuge for multiplication of BHK cells in suspension and for replication of FMD virus. *Bull. Off. Int. Epiz.* 79, 805-822.
21. Girard, H. C., Sureau, M., Erdem, H., and Gurhan, I. (1980). Monolayer cultures of animal cells with the Gyrogen equipped with tubes. *Biotechnol. Bioeng.* 22, 477-493.
22. Gori, G. B. (1965). Chemostatic concentrated cultures of heteroploid mammalian cell suspensions in dialyzing fermenters. *Appl. Microbiol.* 13, 93-97.
23. Graff, S., and McGarry, K. S. (1957). Sustained cell culture. *Exp. Cell Res.* 13, 348-357.
24. Graff, S., and McGarry, K. S. (1959). Energy costs of growth in continuous metazoan cell cultures. *Cancer Res.* 19, 741-746.
25. Griffiths, J. B., Sargeant, K., and Whitaker, A. (1967). The use of the Graff cyrogenerator to grow γ BHK cells. *MRE Record* No. 17.
26. Griffiths, J. B., Thornton, B., and McEneaney, L. (1982). The development and use of microcarrier and glass sphere culture techniques for the production of Herpes simplex virus. Type 2, in MRC-5 cells. *Dev. Biol. Stand.* 50, 103-110.
27. Griffiths, J. B., and Thornton, B. (1982). Use of microcarrier culture for the production of Herpes simplex virus (type 2) in MRC-5 cells. *J. Chem. Technol. Biotechnol.* 32, 324-329.
28. Griffiths, J. B., Atkinson, A., Electricwala, A., Later, A., McEneaney, L., Riley, P. A., and Sutton, P. M. (1984). Production of a fibrinolytic enzyme from cultures of guinea pig keratocytes grown on microcarriers. *Dev. Biol. Stand.* 55, 31-36.
29. Griffiths, J. B., and Riley, P. A. (1985). Cell biology: basic concepts. In "Animal Cell Biotechnology" (R. E. Spier and J. B. Griffiths, eds.), Vol. 1, pp. 17-48. Academic Press, London.
30. Griffiths, J. B. (1986). Scaling-up of animal cell cultures. In "Animal Cell Culture: A Practical Approach" (R. I. Freshney, ed.), Chapter 3, pp. 33-70. IRL Press, Oxford.
31. Griffiths, J. B. (1986). Can cell culture medium costs be reduced? Strategies and possibilities. *Trends Biotechnol.* 4, 268-272.
32. Griffiths, J. B., and Looby, D. (1987). A comparison of oxygenation methods in a 40L stirred bioreactor. "Proceedings 8th ESACT Meeting, Israel". Butterworths, Guildford.
33. Griffiths, J. B., Cameron, D. R., and Looby, D. (1987). Bulk production of anchorage-dependent cells—comparative studies. In "Plant and Animal Cells: Process Possibilities" (C. Webb and E. Mavuna, eds.), pp. 149-161. Ellis Horwood, Chichester.
34. Griffiths, J. B., and Electricwala, A. (1987). Production of tissue plasminogen activators from animal cells. *Adv. Biochem. Eng. Biotechnol.* 34, 147-166.
35. Griffiths, J. B., Cameron, D. R., and Looby, D. (1987). A comparison of unit process systems for anchorage dependent cells. *Dev. Biol. Stand.* 66, 331-338.
36. Handa, A., Emery, A. N., and Spier, R. E. (1987). On the evaluation of gas-liquid interfacial effects on hybridoma viability in bubble column bioreactors. *Dev. Biol. Stand.* 66, 241-253.
37. Harris, E., and Wendenburg, J. (1978). Large scale perfusion of cells growing on surfaces with automatic gas and medium control. *Cytobiologie* 18, 67-75.

38. Himmelstahl, P., Thayer, P. S., and Martin, H. E. (1969). Spin filter culture: the propagation of mammalian cells in suspension. *Science* 164, 555-557.
39. Hu, W. S., Girard, D. J., and Wang, I. C. (1985). Serial propagation of mammalian cells on microcarriers. *Biotechnol. Bioeng.* 27, 1466-1476.
40. Hu, W. S., and Wang, D. I. C. (1986). Mammalian cell technology: a review from an engineering perspective. In "Mammalian Cell Technology" (W. G. Thilly, ed.), pp. 167-197. Butterworths, Boston.
41. Jensen, M. D. (1981). Production of anchorage-dependent cells—problems and their possible solution. *Biotechnol. Bioeng.* 23, 2703-2716.
42. Karkare, S. B., Phillips, P. G., Burke, D. H., and Dean, R. C. (1985). Continuous production of monoclonal antibodies by chemostatic and immobilized hybridoma culture. In "Large-Scale Mammalian Cell Culture" (J. Feder and W. R. Tolbert, eds.), pp. 127-149. Academic Press, Orlando.
43. Kattiger, H. W. D., Scheiter, W., and Kromer, E. (1979). Bubble column reactor for mass propagation of animal cells in suspension culture. *Ger. Chem. Eng.* 2, 31-38.
44. Kattiger, H. W. D., and Scheiter, W. (1982). Status and developments of animal cell technology using suspension culture techniques. *Acta Biotechnologica* 2, 3-41.
45. Kattiger, H. W. D., and Blein, R. (1983). Production of enzymes and hormones by mammalian cell cultures. *Adv. Biotech. Proc.* 2, 61-95.
46. Kattiger, H. W. D. (1987). Principles of animal cell fermentation. *Dev. Biol. Stand.* 66, 195-209.
47. Kizano, K., Shinami, Y., Ichimori, Y., Tsukamoto, K., Sasaki, S., and Kida, M. (1986). Production of human monoclonal antibodies by heterohybridomas. *Appl. Microbiol. Biotechnol.* 24, 282-286.
48. Klement, G., Scheiter, W., and Kattiger, H. W. D. (1987). Construction of a large scale membrane reactor system with different compartments for cells, medium and product. *Dev. Biol. Stand.* 66, 221-226.
49. Knazek, R. A., Guillou, P. M., Kohler, P. O., and Dandrick, R. L. (1972). Cell culture on artificial capillaries: an approach in tissue growth *in vitro*. *Science* 178, 65-67.
50. Koshino, W. (1987). Growth and antibody production in a flexible plastic culture vessel. *In Vitro* 23(3), 22A (Abstract 44).
51. Kuse, P. F., Kelen, L. N., and Whittle, W. L. (1970). Some distinctive characteristics of high density perfusion cultures of diverse cell types. *In Vitro* 6, 75-88.
52. Kuse, P. F., and Medema, E. (1965). Production and characterization of multiple-layered populations of animal cells. *J. Cell. Biol.* 27, 273-279.
53. Kruse, P. F., Whittle, W. L., and Medema, E. (1969). Mitotic and non-mitotic multiple-layered perfusion cultures. *J. Cell. Biol.* 42, 113-121.
54. Ku, K., Kuo, M. J., Delenai, J., Wildi, B. S., and Feder, J. (1981). Development of a hollow fibre system for large-scale culture of mammalian cells. *Biotechnol. Bioeng.* 23, 79-95.
55. Kuhlmann, W. (1987). Optimization of a membrane oxygenation system for cell culture in stirred tank reactors. *Dev. Biol. Stand.* 66, 263-268.
56. Larsson, B., and Litwin, J. (1987). The growth of polio virus in human diploid fibroblasts grown with cellulose microcarriers in suspension. *Dev. Biol. Stand.* 66, 385-390.
57. Lazari, A., Heuvel, S., Mizrahi, A., Avshalon, M., Whiteside, J. P., and Spier, R. E. (1987). Production of biologicals by animal cells immobilized on polyurethane foam matrix. "Proceedings of 8th ESACT meeting, Israel". Butterworths, Guildford.
58. Leist, C., Meyer, H. P., and Fiechter, A. (1986). Process control during the suspension culture of a human melanoma cell line in a mechanically stirred loop bioreactor. *J. Biotechnol.* 4, 235-246.

59. Lehmann, J., Pichl, G. W., and Schulz, R. (1987). Bubble free cell culture aeration with porous moving membranes. *Dev. Biol. Stand.* 66, 227-240.
60. Lim, F., and Sun, A. M. (1980). Microencapsulated islets as bioartificial endocrine pancreas. *Science* 210, 908-910.
61. Linderer, E., Arvidsson, A. C., Wergeland, L., and Billig, D. (1987). Subpassaging cells on microcarriers: the importance for scaling up to production. *Dev. Biol. Stand.* 66, 299-305.
62. Litwin, J. (1985). The growth of human diploid fibroblasts aggregates with cellulose fibres in suspension. *Dev. Biol. Stand.* 69, 237-242.
63. Looby, D., and Griffiths, J. B. (1987). Optimisation of glass-sphere immobilised bed cultures. "Proceedings of 8th ESACT meeting, Israel." Butterworths, Guildford.
64. McCoy, T. A., Whittle, W., and Conway, E. (1962). A glass helix perfusion chamber for massive growth of cells *in vitro*. *Proc. Soc. Exp. Biol. Med.* 109, 235-237.
65. Mann, G. F. (1977). Development of a perfusion culture system for production of biologicals using contact dependent cells. *Dev. Biol. Stand.* 37, 149-152.
66. Miltenberger, H. G., and David, P. (1980). Mass production of insect cells in suspension. *Dev. Biol. Stand.* 46, 183-186.
67. Molin, O., and Heden, C. G. (1969). Large scale cultivation of human diploid cells on titanium discs in a special apparatus. *Prog. Immunobiol. Stand.* 3, 106-110.
68. Montagnon, B., Vincent-Falquet, J. C., and Fanger, B. (1984). Thousand litre scale microcarrier culture of Vero cells for killed Polio virus vaccine. *Dev. Biol. Stand.* 55, 37-42.
69. Munder, P. G., Maddrell, M., and Wallach, D. F. H. (1971). Cell propagation on films of polymer fluorocarbon as a means to regulate pericellular pH and PO_2 in cultured monolayers. *FEBS Lett.* 15, 191-195.
70. Nilsson, K., and Moshach, K. (1980). Preparation of immobilised animal cells. *FEBS Lett.* 186(1), 145.
71. Nilsson, K., Scheiter, W., Merren, O.-W., Osterberg, L., Lihl, E., Kaininger, H. W. D., and Moshach, K. (1983). Entrapment of animal cells for production of monoclonal antibodies and other biomolecules. *Nature (London)* 66, 183-193.
72. Nilsson, K., Buzsaky, E., and Moshach, K. (1986). Growth of anchorage-dependent cells on macroporous microcarriers. *BioTechnology* 4, 989-990.
73. Nilsson, K., and Moshach, K. (1987). Immobilised animal cells. *Dev. Biol. Stand.* 66, 183-193.
74. Nilsson, K. (1987). Methods for immobilizing animal cells. *Trends Biotechnol.* 5, 73-78.
75. Reuveny, S., Zheng, Z.-B., and Epstein, L. (1986). Evaluation of a cell culture fermenter. *Am. Biotechnol. Lab.* Feb., 28-36.
76. Reuveny, S., Velez, D., Miller, L., and MacMillan, J. D. (1986). Comparison of cell propagation methods for their effect on monoclonal antibody yield in fermenters. *J. Immunol. Methods* 86, 61-69.
77. Robinson, J. H., Butlin, P. M., and Imitre, R. C. (1979). Growth characteristics of human diploid fibroblasts in packed beds of glass beads. *Dev. Biol. Stand.* 49, 173-181.
78. Rose, C. G. (1967). The circumfusion system for multipurpose culture chambers. *J. Cell. Biol.* 32, 89-112.
79. Rousseau, L., and BuLock, J. D. (1980). Mixing characteristics of a simple air-lift. *Biotechnol. Lett.* 2, 475-480.
80. Rupp, R. G. (1985). Use of cellular microencapsulation in large-scale production of monoclonal antibodies. In "Large-Scale Mammalian Cell Culture" (J. Feder and W. R. Tolbert, eds.), pp. 19-38. Academic Press, Orlando.
81. Santero, G. G. (1972). The rotary column method for growth of large-scale quantities of cell monolayers. *Biotechnol. Bioeng.* 14, 753-775.
82. Sato, S., Kawamura, K., and Iijiyoshi, N. (1983). Animal cell cultivation for production of

7. Cell Culture Systems and their Scale-up

- biological substances with a novel perfusion culture apparatus. *J. Tissue Cult. Methods* 8, 167-171.
83. Schleicher, J. B., and Weiss, R. E. (1968). Application of a multisurface tissue culture propagator for the production of cell monolayers, virus and biochemicals. *Biotechnol. Bioeng.* 10, 617-624.
84. Schönher, O. T., van Gelder, P. T. J. A., van Hest, P. J., van Os, A. M. J. M., and Koelefs, H. W. M. (1987). A hollow fibre dialysis system for *in vitro* production of monoclonal antibodies replacing *in vivo* production in mice. *Dev. Biol. Stand.* 66, 211-220.
85. Spier, R. E., and Clarke, J. B. (1980). Variation in the susceptibility of BHK populations and cloned cell lines to three strains of FMD virus. *Arch. Virol.* 63, 1-9.
86. Spier, R. E., and Griffiths, J. B. (1983). An examination of the data and concepts germane to the oxygenation of cultured animal cells. *Dev. Biol. Stand.* 55, 81-92.
87. Spier, R. E. (1985). Monolayer growth systems: heterogeneous unit processes. In "Animal Cell Biotechnology" (R. E. Spier and J. B. Griffiths, eds.), Vol. 1, pp. 243-263. Academic Press, London.
88. Spier, R. E., and Whiteside, J. P. (1983). The description of a device which facilitates the oxygenation of microcarrier cultures. *Dev. Biol. Stand.* 55, 151-152.
89. Spier, R. E., and McCullough, K. (1987). The large-scale production of monoclonal antibodies *in vitro*. Cambridge University Press. (In preparation.)
90. Strad, M., Quarles, J. M., and McConnell, S. (1984). A modified matrix perfusion-microcarrier bead cell culture system. *Biotechnol. Bioeng.* 26, 503-507.
91. Tharakan, J. P., and Chau, P. C. (1986). Operation and pressure distribution of immobilized cell hollow fiber bioreactors. *Biotechnol. Bioeng.* 28, 1064-1071.
92. Tharakan, J. P., and Chau, P. C. (1986). A radial flow hollow fiber reactor for the large-scale culture of mammalian cells. *Biotechnol. Bioeng.* 28, 329-342.
93. Tharakan, J. P., and Chau, P. C. (1987). Modeling and analysis of radial flow cell culture. *Biotechnol. Bioeng.* 29, 657-671.
94. Thayer, P. S. (1973). Spin filter device for suspension cultures. In "Tissue Culture Methods and Applications" (P. K. Kruse and M. K. Patterson, eds.), pp. 345-351. Academic Press, New York and London.
95. Tolbert, W. R., Feder, J., and Kimes, R. C. (1981). Large-scale rotating filter perfusion system for high density growth of mammalian suspension cultures. *In Vitro* 17, 885-890.
96. Tolbert, W. R., White, P. J., and Feder, J. (1985). Perfusion culture systems for production of mammalian cell biomolecules. In "Large-Scale Mammalian Cell Culture" (J. Feder and W. R. Tolbert, eds.), pp. 1-18. Academic Press, Orlando.
97. Tovey, M. G. (1985). The cultivation of animal cells in continuous-flow culture. In "Animal Cell Biotechnology" (R. E. Spier and J. B. Griffiths, eds.), Vol. 1, pp. 195-210. Academic Press, London.
98. van Hemert, P. A., Kilburn, D. G., and van Wezel, A. L. (1969). Homogeneous cultivation of animal cells for the production of virus and virus products. *Biotechnol. Bioeng.* 11, 875-881.
99. van Wezel, A. L. (1967). Growth of cell strains and primary cells on microcarriers in homogeneous culture. *Nature (London)* 216, 64-65.
100. van Wezel, A. L. (1982). Cultivation of anchorage-dependent cells and their applications. *J. Chem. Tech. Biotechnol.* 32, 318-323.
101. van Wezel, A. L. (1984). Microcarrier technology—present status and prospects. *Dev. Biol. Stand.* 55, 3-9.
102. van Wezel, A. L., van der Velden-de Groot, C. A. M., de Haan, H. H., van den Heuvel, N., and Schaafsma, R. (1985). Large scale animal cell cultivation for production of cellular biologicals. *Dev. Biol. Stand.* 60, 229-236.

103. Varccka, R., and Scheiter, W. (1987). Use of a rotary wire cage for retention of animal cells in a perfusion fermenter. *Dev. Biol. Stand.* 66, 269-272.
104. Whiteside, J. P., and Spier, R. E. (1981). The scale-up from 0.1 to 100 litre of a unit process system based on 3 mm diameter glass spheres for the production of four strains of FMDV from BHK monolayer cells. *Biotechnol. Bioeng.* 23, 551-565.
105. Whiteside, J. P., Farmer, S., and Spier, R. E. (1985). The use of caged aeration for the growth of animal cells on microcarriers. *Dev. Biol. Stand.* 69, 283-290.
106. Whiteside, J. P., and Spier, R. E. (1985). Factors affecting the productivity of glass sphere propagators. *Dev. Biol. Stand.* 69, 305-311.
107. Wohler, W., Rudiger, H. W. & Passarge, E. (1972). Large scale culturing of normal diploid cells on glass beads using a novel type of culture vessel. *Exp. Cell Res.* 74, 571-573.
108. WHO (1987). Acceptability of cell substrates for production of biologicals. Technical Report Series No. 747.
109. Amicon Corporation, Danvers, MA 01923, U.S.A. Technical publication (402).
110. APV International Ltd., Crawley RH10 2QB, U.K. Plate heat exchanger.
111. Bellco Glass Inc., Vineland, NJ 08360, U.S.A. U-carrier magnetic stirrer.
112. Bellco Glass Inc., Vineland, NJ 08360, U.S.A. Bellco-Conbell Alternator Roller Culture Apparatus.
113. Bioengineering AG., CH 8636 Waid, Switzerland. Membrane Laboratory Fermenter and Fluidised Bed Reactor.
114. Edmund Buhler, D-7400 Tübingen, F.R. Germany. Buhler Cell Culture System ZKA.
115. Endotronics Inc., Coon Rapids, MN 55433, U.S.A. Acusyst hollow fibre reactors.
116. J. R. Scientific Inc., Woodland, CA 95695, U.S.A. Media supplied in plastic bags up to 500 litres volume.
117. LH Fermentation, Stoke Poges SL2 4EG, U.K. Spin exchanger filter.
118. New Brunswick Scientific, Edison NJ 08818, U.S.A. Perfusion swirl cap roller bottles.
119. SGI, 31100 Toulouse, France. Cellascence Impeller.
120. Tecne (Cambridge) Ltd., Duxford, U.K. MCS series of biological stirrers.
121. Tecne (Cambridge) Ltd., Duxford, U.K. BR-06 Bioreactor.
122. Rohm and Haas Co., Philadelphia, PA 19105, U.S.A. Biocryl BPA Bioprocessing aids.

8

Bubble-free Reactors and Their Development for Continuous Culture with Cell Recycle

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44

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Office Action Summary

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Applicant(s)

ZHANG ET AL.

Examiner

Shanon A. Foley

Art Unit

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-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE ____ MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133).
- Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 22 June 2001.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-43 is/are pending in the application.
- 4a) Of the above claim(s) 30-43 is/are withdrawn from consideration.
- 5) ☐ Claim(s) ____ is/are allowed.
- 6) ☒ Claim(s) 1-29 is/are rejected.
- 7) ☐ Claim(s) ____ is/are objected to.
- 8) ☐ Claim(s) ____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on ____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
- Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
- 11) ☐ The proposed drawing correction filed on ____ is: a) ☐ approved b) ☐ disapproved by the Examiner.
- If approved, corrected drawings are required in reply to this Office action.
- 12) ☐ The oath or declaration is objected to by the Examiner.

Priority under 35 U.S.C. §§ 119 and 120

- 13) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. ____.
3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
- * See the attached detailed Office action for a list of the certified copies not received.
- 14) ☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. § 119(e) (to a provisional application).
- a) ☐ The translation of the foreign language provisional application has been received.
- 15) ☒ Acknowledgment is made of a claim for domestic priority under 35 U.S.C. §§ 120 and/or 121.

Attachment(s)

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☒ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) ☒ Information Disclosure Statement(s) (PTO-1449) Paper No(s) 4.
- 4) ☐ Interview Summary (PTO-413) Paper No(s). ____.
- 5) ☐ Notice of Informal Patent Application (PTO-152)
- 6) ☐ Other: _____

Art Unit: 1648

DETAILED ACTION

The examiner of your application has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Group Art Unit 1648, Examiner Foley.

Election/Restrictions

Applicant's election without traverse of Group I, claims 1-29 in Paper No. 16 is acknowledged.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Claim 29 is provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 31 and 43 of copending Application No. 09/880,609. Although the conflicting claims are not identical, they are not patentably distinct from each other because the adenovirus obtained by the process claimed in the instant application is indistinguishable from the adenovirus contained in the composition in application '609.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Claims 1-29 are rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-5, 9, 11-18, 20, 21, 30-39, 41-47, 50-53, 61-64, 66-69, 71-74, 78-80, 86-89 of U.S. Patent No. 6,194,191. Although the conflicting claims are not identical, they are not patentably distinct from each other because although the method of preparing the adenovirus in the patent does not require that the producer cells be infected at a particular phase in the cell cycle, a cell culture comprises cells in all phases of the cycle. Therefore, the patent inherently covers cells that are infected at every specific phase, even though it is not specifically claimed.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Claims 30-43 of this application conflict with claims 30-43 of Application No. 09/880,609. 37 CFR 1.78(b) provides that when two or more applications filed by the same applicant contain conflicting claims, elimination of such claims from all but one application may be required in the absence of good and sufficient reason for their retention during pendency in more than one application. Applicant is required to either cancel the conflicting claims from all but one application or maintain a clear line of demarcation between the applications. See MPEP § 822.

A rejection based on double patenting of the "same invention" type finds its support in the language of 35 U.S.C. 101 which states that "whoever invents or discovers any new and useful process ... may obtain a patent therefor ..." (Emphasis added). Thus, the term "same invention," in this context, means an invention drawn to identical subject matter. See *Miller v. Eagle Mfg. Co.*, 151 U.S. 186 (1894); *In re Ockert*, 245 F.2d 467, 114 USPQ 330 (CCPA 1957); and *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970).

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A statutory type (35 U.S.C. 101) double patenting rejection can be overcome by canceling or amending the conflicting claims so they are no longer coextensive in scope. The filing of a terminal disclaimer cannot overcome a double patenting rejection based upon 35 U.S.C. 101.

Claims 30-43 are provisionally rejected under 35 U.S.C. 101 as claiming the same invention as that of claims 30-43 of copending Application No. 09/880,609. This is a provisional double patenting rejection since the conflicting claims have not in fact been patented.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 3, 4, 20, 23, and 25 are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 3 is drawn to producer cells being seeded using a homogeneous pool of cells. How are the homogeneous cells aiding the seeding of the producer cells? Is the homogeneity of the cells referring to cell type and/or synchronicity of the cell phase? If the producer cells are a homogeneous pool of cells in the same cycle, it is suggested that applicant amend the claim to reflect this concept.

Claim 4 states that the producer cells are perfused "at least a portion of the time". Are the producer cells being perfused in step a) of claim 1, and not step b), or vice versa? Or are the cells being perfused off and on in steps a) and b) of claim 1? It cannot be discerned what is meant by this phrase in the claim since the specification defines perfusion as a continuous process, see page 31, line 11.

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Claim 20 recites the limitation "producer cells" in line 1. There is insufficient antecedent basis for this limitation in the claim. For examining purposes, this claim is being treated as if it depends from claim 1. However, this does not relieve applicant of the burden of response to this rejection.

Claim 23 recites the limitation "recombinant gene" in line 1. There is insufficient antecedent basis for this limitation in the claim.

Claim 25 recites the limitation "said promoter" in line 1. There is insufficient antecedent basis for this limitation in the claim.

The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Claim 26-28 are rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Claim 26 is drawn to lysing producer cells "by means other than freeze-thaw". While the specification on page 35, line 23 –page 37, and page 42, lines 9-12, discusses a variety of methods to lyse cells, including freeze-thaw, the specification does not reasonably support the specific exclusion of the freeze-thaw method. Although Table 2 on page 37 does identify this method as "not scalable, not recommended for large scale manufacturing", the table also identifies other methods as "scalability concerns". Although the specification reasonably conveys a variety of methods for lysing cells, the specification is not seen as reasonably

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conveying the concept of “any method except freeze-thaw”, which is now the scope of the subject matter of these claims. This affects dependent claims 27 and 28.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

Claims 1, 2, 9, and 13-24, and 26 are rejected under 35 U.S.C. 102(b) as being anticipated by Huyghe et al. (C44).

The claims are drawn to a process of preparing a p53 recombinant adenovirus by preparing a culture of 293 cells, and infecting the cells between mid-log and stationary phase and, harvesting the adenovirus from the culture by a variety of chromatographic means, including ion exchange chromatography, and putting the harvested virus into a pharmaceutical composition.

Huyghe et al. teaches a method for purifying a recombinant adenovirus encoding p53 from 293 cells by a variety of chromatographic techniques, including ion exchange, see the abstract, first paragraph under “materials and methods”, and the section bridging pages 1407-1408. Since the cells were infected at 50-60% confluency, the cell culture would inherently comprise cells at every phase in the cell cycle at the time of infection and therefore anticipate infecting cells at a particular phase.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claim 3 is rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al. as applied to claims 1, 2, 9, and 13-24, and 26 above, and further in view of Kraft et al. (Archives of Virology 1978. 57 (3): 243-54. Abstract only.)

Although this claim is indefinite for reasons discussed above, in the interest of compact prosecution, it is assumed that the claim is drawn to the producer cells being homogeneous pool of cells of the same type and phase. Huyghe et al. does not teach this concept. However, Kraft et al. correlates the production of CELO adenovirus with the S phase in synchronized cultures. Therefore, it would have been obvious for one of ordinary skill in the art to infect a homogeneously synchronized cell culture at the optimum time in the cell cycle to increase virus yields. One of ordinary skill in the art at the time the invention was made would have had a reasonable expectation in producing the claimed invention by the routine optimization technique taught by Kraft et al. Therefore, the invention is seen as prima facie obvious, absent unexpected results.

Claims 4-7 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al. as applied to claims 1, 2, 9, and 13-24, and 26 above, and further in view of Garnier et al. (C26) or Perrin et al. (C73).

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The claims are drawn perfusing the producer cells to maintain a certain level of glucose in the culture. Huyghe et al. does not teach perusing the 293 cells. However, in the art of virus production, perfusion cultures have been used for large-scale growth of cells for virus production. For example, Perrin et al. and Garnier et al. teach scale-up adenovirus growth using medium replacement for controlling glucose concentrations for improved virus yields. It would have been within the skill of the ordinary artisan to scale-up culture using a perfusion system for the advantages of large-scale production of virus as suggested by Perrin et al., and to optimize the rate of medium replacement and glucose level for the advantage of improving virus yield taught by Garnier et al. Therefore, the invention is seen as *prima facie* obvious, absent unexpected results.

Claim 27 is rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al., Garnier et al., and Perrin et al. as applied to claims 1, 2, 4-7, 9, 13-24, and 26 above, and further in view of Graham et al. (C32).

The claim is drawn to lysing the producer cells by detergent lysis. Huyghe et al., Garnier et al., nor Perrin et al. teach lysing cells with detergent. Graham et al. teaches that using a 5% sodium deoxycholate will disrupt cells without disrupting adenovirus virions, see page 119. Therefore, it would have been *prima facie* obvious to use a detergent as an alternative method to lyse adenovirus-infected cells.

Claims 8 and 25 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al., Kraft et al., Garnier et al., Perrin et al., or Graham et al. as applied to claims 1-7, 9, 13-24, 26 and 27 above, and further in view of Zhang et al. (6,143,290).

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The claims are drawn to a step of cell settling before infection and the p53 gene under the control of a particular promoter.

None of the previous references teach the specific promoter, but Zhang et al. teaches a method for expressing an adenovirus encoding p53 that is under the control of an SV40 promoter. Since all of the promoters listed in claim 25 are used conventionally for gene expression in adenovirus, all would be obvious alternatives to the promoter used by Zhang et al. to one of ordinary skill in the art at the time the invention was made.

Zhang et al. also teaches that the 293 cells were inoculated 24 hours prior to viral transfection into flasks, see column 18, lines 12-18. In the tissue culture art, it is standard practice to let cells settle and recover at least a few hours before manipulation. One of ordinary skill in the art at the time the invention was made would have been motivated to get the producer cells in optimum condition before transfection, i.e. letting them settle. Immediately burdening the cells with more stress from the virus to be transfected would decrease cell survival and lead to low virus yields. Therefore the invention as a whole would have been prima facie obvious to the ordinary artisan, absent unexpected results.

Claims 10-12 and 29 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al., Kraft et al., Garnier et al., Perrin et al., Graham et al., or Zhang et al. as applied to claims 1-9 and 13-27.

The claims are drawn to specific producer cell numbers to be plated prior to transfection and particular characteristics of the harvested adenovirus

All of the references teach various methods of purifying recombinant adenoviruses. Although none of the references teach specific cell numbers to be plated, this number would be a

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very subjective determination by one of ordinary skill in the art to be based on many different factors, such as the type of cell, what condition the cells are in before plating, how fast the cells divide, ect. All of these factors and others must be taken into account before seeding. Too many cells will result in clumping and the virus will not have access to as many cells as it would with fewer cells. Too few cells would result in poor cell condition from lack of other cell contact and eventually cell death (depending on the type of cell). Therefore, it would be prima facie obvious for one of ordinary skill in the art to determine the appropriate cell number required for each situation encountered.

Although none of the references teach a harvested adenovirus with the characteristics listed in claim 29, all of the references teach various methods for improving the quantity and/or purity of the recombinant adenovirus obtained. Therefore, it would be obvious for one of ordinary skill in the art at the time the invention was made to test for any one of the properties listed to ensure a good yield of adenovirus.

The invention as a whole would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made, absent unexpected results.

Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Shanon A. Foley whose telephone number is (703) 308-3983. The examiner can normally be reached on 7:30-4:30 M-F.

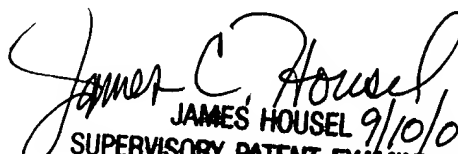
If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James Housel can be reached on (703) 308-4027. The fax phone numbers for the

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organization where this application or proceeding is assigned are (703) 308-4242 for regular communications and (703) 308-4426 for After Final communications.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the receptionist whose telephone number is (703) 308-0196.

Shanon Foley/SAF
September 7, 2001


JAMES HOUSEL 9/10/01
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/203,078	12/01/1998	SHUYUAN ZHANG	INRP:081	3754
7590 06/03/2004			EXAMINER	
STEVEN L. HIGHLANDER FULBRIGHT & JAWORSKI L.L.P. 600 CONGRESS AVENUE, SUITE 2400 AUSTIN, TX 78701			FOLEY, SHANON A	
			ART UNIT	PAPER NUMBER
			1648	

DATE MAILED: 06/03/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

RECEIVED

3-month response date - 09-03-04
Final deadline for 3-month OA response - 12-03-04

JUN 03 2004

Client: INRP: 081

Attorney(s): SLH/MRK/GNS

Initials: dw

10012499

Office Action Summary

Application No.

09/203,078

Applicant(s)

ZHANG ET AL.

Examiner

Shanon Foley

Art Unit

1648

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 08 March 2004.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-62 is/are pending in the application.
- 4a) Of the above claim(s) 33-37 is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 1-32 and 38-62 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
 2. ☐ Certified copies of the priority documents have been received in Application No. _____.
 3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).
- * See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
- 3) ☐ Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08)
Paper No(s)/Mail Date _____.
- 4) ☐ Interview Summary (PTO-413)
Paper No(s)/Mail Date. _____.
- 5) ☐ Notice of Informal Patent Application (PTO-152)
- 6) ☐ Other: _____.

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DETAILED ACTION

In the amendment submitted March 8, 2004, applicant amended claims 1-3, 18, 29 and added new claims 30-62. Claims 1-62 are pending.

Upon further consideration of the claims and the prior art and a reconsideration of the declaration under 37 CFR 1.132 by Shawn Gallagher submitted February 28, 2002, it is determined that new grounds of rejection are required.

Election/Restrictions

Newly submitted claims 33-37 are directed to an invention that is independent or distinct from the invention originally claimed for the following reasons: The claims encompass administration of the adenovirus. The subject matter of the claims under examination is drawn to a process of preparing an adenovirus. The active steps of administering in new claims 33-37 do not further limit steps of preparing. Therefore, the subject matter encompassed by claims 33-37 are beyond the scope of the originally presented claims.

Since applicant has received an action on the merits for the originally presented invention, this invention has been constructively elected by original presentation for prosecution on the merits. Accordingly, claims 33-37 are withdrawn from consideration as being directed to a non-elected invention. See 37 CFR 1.142(b) and MPEP § 821.03.

Claims 1-32 and 38-62 are under consideration.

Priority

From the "Remarks" section, applicant appears to intend the first line of the specification to disclaim benefit of priority to parent applications. However, the actual amendment to the specification cites page 3, instead of page 2. Therefore, while the examiner acknowledges the

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cancellation of priority, an appropriate correction to the amendment of the specification is required. In view of the amendment of the priority claimed, the effective date of the instant application is the filing date, December 1, 1998.

Claim Rejections - 35 USC § 112

The following is a quotation of the second paragraph of 35 U.S.C. 112:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

Claims 32 and 48 is rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 32 is incomprehensible. Is the pharmaceutically acceptable composition different from the pharmaceutically acceptable carrier?

It cannot be determined if the improvement of claim 48 requires the improvement of claim 47 or not. It is presumed that the improvement recited in claim 48 is additional to the limitations of claim 47. However, this presumption does not relieve applicant of the necessity to clarify the intended limitations of the claim. This rejection could be obviated by the insertion of "further" after "improvement" in line 1.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground

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provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Since the priority date of this application has changed, US 2002/018723 A1 is now available as prior art since the subject matter claimed in US 2002/018723 A1 is supported in an application with a filing date of November 20, 1997.

Claims 1, 3-29, 38 and 47-62 are provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-29 of copending Pre-grant Publication Patent Application No. US 2002/018723 A1. Although the conflicting claims are not identical, they are not patentably distinct from each other because the instant claims encompass preparing a recombinant adenovirus, as evidenced by claim 38 for example. The remaining limitations between the instant claims and the pre-grant application publication are the same.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an

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international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claims 1, 3, 8, 9, 13-25, 31, 32, 38, 47, 49 and 51-62 are rejected under 35 U.S.C. 102(b) as being anticipated by Huyghe et al. (Human Gene Therapy. 1995; 6: 1403-1416) in light of Kuchler, previously provided by applicant.

Applicant reiterates the broadest claim on page 12 and asserts that the summary provided is incorrectly characterized. Applicant's reiteration of the claim is appreciated, but the summary of the rejected claims starting on page 2 of the previous Office action includes the limitations of several claims for the sake of efficiency and succinctness. The teaching of a more limiting factor in the prior art of the claims necessarily teaches the broader concepts in less limiting claims. Therefore, the collection of claims anticipated by Huyghe et al. are summarized as follows:

The claims are drawn to a process of preparing adenovirus by preparing a culture of producer cells essentially homogenous with respect to growth, infecting the producer cells with adenovirus between mid-log and stationary phase, and harvesting the adenovirus and placing the virus into a pharmaceutically acceptable composition. The cells are allowed to attach to a surface between 3 and 24 hours prior to infection and are recirculated during infection. The adenovirus is replication-defective, lacking a portion of E1, which is complemented by 293 cells, and encodes the p53 gene from a CMV promoter. The adenovirus is purified by only one or several chromatographic separations including ion-exchange chromatography.

Huyghe et al. anticipate preparing adenovirus by preparing a culture of 293 producer cells that have attained an essentially homogenous confluency of 50-60% when the cells are infected with a replication-defective adenovirus expressing p53 from a CMV promoter in place of E1

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coding sequences. This percentage of confluency reasonably corresponds to mid-log phase of cell growth (explained in greater detail below). The 293 cells are allowed to attach to the surface between 2 and 2.5 days prior to infection and upon infection, the virus was mixed thoroughly with the cell culture medium. The adenovirus is harvested and added to phosphate-buffered saline supplemented with 2% sucrose and 2 mM MgCl_2 , a pharmaceutically acceptable carrier. The adenovirus of Huyghe et al. is purified by several methods of chromatography, including ion-exchange chromatography. See the first full paragraph of the second column on page 1403, "Production of infected ATCC 293 cells", "Harvest and lysis", "Preparation of ACN53 standard material" and "Chromatographic parameters" bridging pages 1404-1405.

In the response and the declaration by Shawn Gallagher submitted February 28, 2002, applicant assumed seeding densities of Huyghe et al. and provided reasons for how the assumed seeding densities would be consistent the early log phase of growth. However, the Freshney reference and the Mediatech Technical Information provided with the declaration clearly indicate a number of factors contributing to the length of the log phase, which include seeding density and changes in the growth medium, see "The Log Phase" of Freshney on page 239 and "Growth Phases" in Mediatech Technical Information. Since seeding density is established in the art as a crucial component of the log phase and Huyghe et al. has not provided any information regarding the initial density of cells, applicant's presumption of early log phase density for the cells of Huyghe et al. is speculative and unsubstantiated.

When applying the teachings of Freshney to the cell density of Huyghe et al., applicant reasoned that the cells of Huyghe et al. would not be in late log phase and the examiner agrees.

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With respect to the teachings of Mediatech's Technical Information, applicant equates 70% confluency with log phase and reasons that 50%-60% confluency is only in early log phase at the very most. However, applicant appears to have misinterpreted the reference. While the reference indicates that cultures that are 70% confluent are in log phase, there is no differentiation between the various stages of log growth and % confluency provided by the reference. Therefore, applicant's conclusion that 50-60% confluency equates to early log phase is unsupported.

Finally, when the teachings of Kuchler are applied to the cells of Huyghe et al., applicant determines that the cells of Huyghe et al. are barely out of lag phase since the lag times of 293 cells ranges between 24-48 hours and Huyghe et al. infected the cells between 48 and 60 hours after seeding. Therefore, the supported facts provided by the references and the declaration are:

- 1) the lag time of 293 cells ranges between 24-48 hours
- 2) the cells of Huyghe et al. are have a confluency of 50-60% upon infection
- 3) the cells of Huyghe et al. attach to the surface of the plate for 48 to 60 hours before infection, which is beyond the hours required for the lag phase
- 4) the chart provided by Kuchler indicates that the growth curve of cells after 60 hours of incubation is the mid-point of the growth curve, i.e. mid-phase.

Therefore, from the factual evidence available, it is determined that the cells of Huyghe et al. are at mid-phase upon infection.

Claims 1, 3-9, 13-28, 30-32, 38-49 and 51-62 are rejected under 35 U.S.C. 102(a) as being anticipated by Zhang et al. (WO 98/22588), as further evidenced by Wu et al. (US 6,689,600 B1).

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Claims 1, 3-9, 13-28, 30-32, 38-49 and 51-62 are rejected under 35 U.S.C. 102(e) as being anticipated by Zhang et al. (US Patent No. 6,194,191 B1), as further evidenced by Wu et al. (US 6,689,600 B1).

The applied reference has a common inventor with the instant application. Based upon the earlier effective U.S. filing date of the reference, it constitutes prior art under 35 U.S.C. 102(e). This rejection under 35 U.S.C. 102(e) might be overcome either by a showing under 37 CFR 1.132 that any invention disclosed but not claimed in the reference was derived from the inventor of this application and is thus not the invention "by another," or by an appropriate showing under 37 CFR 1.131.

Since the priority date of the instant application has changed to December 1, 1998, the disclosures of Zhang et al. (WO 98/22588, published May 28, 1998) and US (6,194,191, which enjoys the benefit of priority to November 20, 1996) are currently available as prior art. Since the disclosures of WO 98/22588 and US 6,194,191 are identical, only citations from the US patent are cited in the rejection in the interest of efficiency.

See the summary of claims 1, 3, 8, 9, 13-25, 29, 31, 32, 38, 47, 49 and 51-62 above. Claims 4-7, 10-12, 26-28, 30, 39-46 and 48 state that the culture cells are perfused for at least a portion of the time at a rate that maintains glucose levels between 1 and 1.5 gm of glucose/liter. The cells are lysed by means other than freeze-thaw. The producer cells are cultured in a various systems including a microcarrier, multiplate, perfused packed bed reactor, microencapsulation or bioreactors, such as stirred tank, airlift or sparge.

Zhang et al. anticipate a process of preparing adenovirus by preparing a culture of producer cells essentially homogenous with respect to growth, infecting the producer cells with

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adenovirus between mid-log and stationary phase, and harvesting the adenovirus and placing the virus into a pharmaceutically acceptable composition. Although Zhang et al. do not specifically teach "mid-log", the cells are determined to be at least mid-phase based on the 80-90% confluence and the types of conditions the cells are cultured in. In addition, Wu et al. provides a summary of the teachings of Zhang et al. and teaches that the cells of Zhang et al. are infected after mid-phase, see column 6, lines 18-34. The cells are allowed to attach to a surface between 3 and 24 hours prior to infection and are recirculated during infection. The adenovirus is replication-defective, lacking a portion of E1, which is complemented by 293 cells, and encodes the p53 gene from a CMV promoter. The adenovirus is purified by only one or several chromatographic separations including ion-exchange chromatography. In addition, Zhang et al. anticipate glucose levels during perfusion between 0.7 and 1.7 g/L and lysing the cells by autolysis or detergent. Zhang et al. also anticipate culturing the producer cells in various systems including a microcarrier, multiplate, perfused packbed reactor, microencapsulation or bioreactors, such as stirred tank, airlift or sparge. See claims column 9, line 43 to column 16, line 49, column 40, line 49 to column 57, line 13 and claims 1-5, 9, 11-18, 20, 21, 28, 29, 30-39, 41-45, 61-63, 66, 68, 70-72, 77-80, 86 and 89.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

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Claims 10-12 and 29 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al. as applied to claims 1, 3, 8, 9, 13-25, 31, 32, 38, 47, 49 and 51-62 above **or** Zhang et al. (WO 98/22588) **or** Zhang et al. US (6,194,191) as applied to claims 1, 3-9, 13-28, 30-32, 38-49 and 51-62.

The claims are drawn to specific seeding densities of the producer cells and particular characteristics of the harvested adenovirus.

Although none of the references, in the alternative, teach the specific cell numbers to be plated, the number would be a subjective determination by one of ordinary skill based on many factors, such as the type of cell, the condition of the cells before plating, and the nature of the cell's division, ect. Therefore, it would be prima facie obvious for one skilled in the art to determine the appropriate number of cells to plate for each situation encountered.

Further, although none of the references, in the alternative, teach a harvested adenovirus with the characteristics listed in claim 29, all of the references teach various methods of improving the quantity and/or purity of the recombinant virus obtained. Therefore, it would have been prima facie obvious to one of ordinary skill to test any one of the properties listed to ensure a good yield of adenovirus.

Claims 2 and 50 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al. as applied to claims 1, 3, 8, 9-12, 13-25, 29, 31, 32, 38, 47, 49 and 51-62 above **or** Zhang et al. (WO 98/22588) **or** Zhang et al. US (6,194,191) as applied to claims 1, 3-32, 38-49 and 51-62 and further in view of Graham et al. (C31 of IDS) and Leu et al. (6,194,210 B1).

Claims 2 and 50 require that the cells are infected in late-log and stationary phase of growth.

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See the teachings of Huyghe et al. or either Zhang et al. reference in the alternative.

None of the references specifically teach infecting at late-log to stationary phase of cell growth.

As stated in the previous Office action, Leu et al. teach a method of producing large quantities of virus by allowing uniform attachment of cells, growing the cells to late-log phase with medium replenishment to provide adequate cell nutrition and infecting the cells at late-log phase and harvesting the virus, see column 11, lines 18-column 12, line 9 and claims 1 and 4.

One of ordinary skill in the art at the time the invention as made would have been motivated to have propagated the adenovirus of Huyghe et al. or either Zhang et al. reference with the cell culture method steps of infection of Leu et al. to increase the amount of adenovirus produced in cell culture. One of ordinary skill in the art at the time the invention was made would have had a reasonable expectation of growing the adenovirus of Huyghe et al. or either Zhang et al. reference with the cell culture method steps taught by Leu et al. because Leu et al. teach that a wide range of viruses may be propagated to generate vaccines using the method steps, see column 5, lines 29-32. Therefore, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art, absent unexpected results to the contrary.

Applicant argues that Leu et al. is replete with references to hepatitis A and argues that the ordinary artisan would not turn to Leu et al. for method steps to culture adenovirus.

Applicant provides a detailed discussion of the differences between hepatitis A and adenoviruses. Applicant also argues that the wide range of viruses mentioned in Leu et al. do not belong to the same family as Adenoviridae.

Applicant's arguments as well as the declaration of Shuyuan Zhang have been fully considered, but are found unpersuasive. As applicant has pointed out, the method of viral

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propagation taught by Leu et al. is clearly applicable to a wide range of unrelated virus families.

Therefore, the teachings of Leu et al. are clearly a teaching applicable to the general viral propagation art.

Applicant also argues that Leu et al. do not teach any benefit of infecting cells at a particular time frame. In response to applicant's argument that the examiner's conclusion of obviousness is based upon improper hindsight reasoning, it must be recognized that any judgment on obviousness is in a sense necessarily a reconstruction based upon hindsight reasoning. But so long as it takes into account only knowledge which was within the level of ordinary skill at the time the claimed invention was made, and does not include knowledge gleaned only from the applicant's disclosure, such a reconstruction is proper. See *In re McLaughlin*, 443 F.2d 1392, 170 USPQ 209 (CCPA 1971).

In the instant case, it has been clearly demonstrated above that Huyghe et al. or either Zhang et al. reference anticipate infecting cells at mid-log phase. Leu et al. specifically teach infecting at late log phase and provide a clear motivation, i.e., to produce large quantities of virus. One of ordinary skill in the art at the time the invention was made would have had a reasonable expectation of success for combining the teachings of Leu et al. with Huyghe et al. or either Zhang et al. reference not only because the method of Leu et al. is applicable to general viral propagation, but also because the primary references teach infection of adenovirus at least at mid-log phase. Further, Mediatech's Technical Information demonstrate that cells of at least 70% confluency are in log-phase. Therefore, cell confluency of 80-90% at the time of infection would certainly be at late-log phase. Graham et al. (reference C31 of the IDS) teaches infecting cells at 80-90% confluency with adenovirus, see section 3.1.2 on page 117. Graham et al. clearly

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demonstrate that the teachings of Leu et al. are applicable to adenovirus infection in cells at late-log phase of growth. Therefore, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art, absent unexpected results to the contrary.

Claims 26-28 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al. as applied to claims 1, 3, 8, 9-12, 13-25, 29, 31, 32, 38, 47, 49 and 51-62 above, and further in view of Graham et al. (C7) for reasons of record.

Applicant argues that Graham et al. do not cure the deficiencies in the primary references. However, there are no deficiencies to cure. Therefore, the rejection is maintained for reasons of record. Further, it is noted that autolysis would be a conventional alternative to detergent lysis.

Claims 4, 30, 39-46 and 48 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al. as applied to claims 1, 3, 8, 9-12, 13-25, 29, 31, 32, 38, 47, 49 and 51-62 above, and further in view of Garnier et al. (C26) and Spier et al. (C35 of the IDS).

The claims require the producer cells to be perfused for a portion of the time the cells are cultured. The claims also require the cells to be cultured in various systems including a microcarrier, multiplate, perfused packed bed reactor, microencapsulation or bioreactors, such as stirred tank, airlift or sparge.

Huyghe et al. do not teach perfusion or the various culture systems recited.

However, Garnier et al. teach scale-up adenovirus growth using medium replacement for controlling glucose concentrations for improved virus yields in a bioreactor, see the material and methods section. One of ordinary skill in the art at the time the invention was made would have been motivated to use the system of Garnier in the method of Huyghe et al. to produce larger quantities of adenovirus. One of ordinary skill in the art at the time the invention was made

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would have had a reasonable expectation of success in combining the teachings of Garnier et al. and Huyghe et al. because both references culture 293 cells for the propagation of adenovirus.

Neither Huyghe et al. nor Garnier et al. teach the various culture systems claimed.

However, Spier et al. review each of the various culture systems claimed, see the entire reference. One of ordinary skill in the art at the time the invention was made would have been motivated to use a conventionally applied culture system, described by Spier et al. in the method and system of Huyghe et al. and Garnier et al. One of ordinary skill in the art at the time the invention was made would have had a reasonable expectation of success in using any of the culture systems of Spier et al. in the method of Garnier et al. and Huyghe et al. because Garnier et al. use a bioreactor system to propagate large quantities of adenovirus and Spier et al. review various types of bioreactor systems. Therefore, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art, absent unexpected results to the contrary.

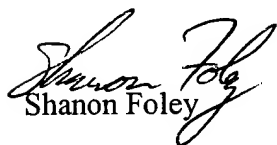
Conclusion

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Shanon Foley whose telephone number is (571) 272-0898. The examiner can normally be reached on M-F 9:30 AM - 6:00 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James Housel can be reached on (571) 272-0902. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

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Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).


Shanon Foley


JAMES HOUSEL
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600
6/1/04



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/203,078	12/01/1998	SHUYUAN ZHANG	INRP:081	3754

7590 11/17/2004

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EXAMINER

FOLEY, SHANON A

ART UNIT

PAPER NUMBER

1648

DATE MAILED: 11/17/2004

Please find below and/or attached an Office communication concerning this application or proceeding.

2 mo. due date to provide
Advisory Action. 11/17/05. Initial
deadline for final OA 2/17/05.

RECEIVED
Date(s) Docketed: <u>11/17/05</u>
<u>Final OA 5/17/05. Initial deadline</u>
<u>for Notice of Appeal 2/17/05. Final</u>
<u>deadline for Notice of Appeal 5/17/05</u>
NOV 22 2004
Client: <u>INRP: 081</u>
Attorney(s): <u>SLH, GNS</u>
Initials: <u>[Signature]</u>

10012499

Office Action Summary

Application No.

09/203,078

Applicant(s)

ZHANG ET AL.

Examiner

Shanon Foley

Art Unit

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-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If the period for reply specified above is less than thirty (30) days, a reply within the statutory minimum of thirty (30) days will be considered timely.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

- 1) ☒ Responsive to communication(s) filed on 07 September 2004.
- 2a) ☒ This action is FINAL. 2b) ☐ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

- 4) ☒ Claim(s) 1-31 and 33-62 is/are pending in the application.
- 4a) Of the above claim(s) 33-37 is/are withdrawn from consideration.
- 5) ☐ Claim(s) _____ is/are allowed.
- 6) ☒ Claim(s) 1-31 and 38-62 is/are rejected.
- 7) ☐ Claim(s) _____ is/are objected to.
- 8) ☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on _____ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some * c) ☐ None of:
- ☐ Certified copies of the priority documents have been received.
 - ☐ Certified copies of the priority documents have been received in Application No. _____.
 - ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

- | | |
|--|---|
| 1) <input type="checkbox"/> Notice of References Cited (PTO-892) | 4) <input type="checkbox"/> Interview Summary (PTO-413) |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948) | Paper No(s)/Mail Date. _____ |
| 3) <input checked="" type="checkbox"/> Information Disclosure Statement(s) (PTO-1449 or PTO/SB/08) | 5) <input type="checkbox"/> Notice of Informal Patent Application (PTO-152) |
| Paper No(s)/Mail Date <u>4/5/4, 7/13/3, 3/27/00</u> . | 6) <input type="checkbox"/> Other: _____ |

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DETAILED ACTION

In the amendment submitted September 7, 2004, applicant amended claim 48 and cancelled claim 32. Claims 1-31 and 33-62 are pending, claims 33-37 are withdrawn from consideration due to nonelected subject matter and claims 1-31 and 38-62 are under consideration.

Upon review of the file history, it appears that some of the references that have previously been submitted by applicant as exhibits, such as Kuchler and Freshney, are incomplete. For example, the Freshney reference only contains the title page and page 239 and the Kuchler reference available for review contains the title page and pages 91, 99 and 100. Since paragraph 10 of Mr. Gallagher's declaration mentions page 90 of Kuchler, it appears that the electronic conversion of the application by the Office did not scan all of the pages of these references. Applicant is requested to supply a copy of all of the references in exhibits again so that the Office file history is complete. Any inconvenience experienced by applicant is regretted.

Information Disclosure Statement

Since the new electronic scanning system at the Office has been implemented, it cannot be determined from our file records if the IDS's submitted April 5, 2004, July 14, 2003 and March 27, 2000 have been considered. In any case, the IDS's have been presently considered or reconsidered and an initialed, signed copy of each is being forwarded to applicant.

Double Patenting

The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. See *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686

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F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and, *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent is shown to be commonly owned with this application. See 37 CFR 1.130(b).

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

In the previous Office action, Pre-grant Publication Patent Application No. US 2002/018723 A1 was applied in a provisional rejection. In response, applicant has supplied a copy of the currently pending claims in the corresponding application no. 09/880,609. Applicant states that the instant claims and the '609 claims are believed to be patentably distinct.

A copy of the '609 claims provided by applicant have been considered. However, claims 30 and 31 of '609 are not considered to be patentably distinct from instant claims 1 and 29 for the reasons previously presented. If claims 30 and 31 of '609 and instant claims 1 and 29 had been filed in the same application, a restriction could not have been drawn between the two sets of claims because the subject matter is not divergent, the process is only able to make the single product claimed and the product claimed can only be made by the single process. Therefore, claims 30 and 31 of '609 and instant claims 1 and 29 are not patentably distinct.

Claim Rejections - 35 USC § 102

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(a) the invention was known or used by others in this country, or patented or described in a printed publication in this or a foreign country, before the invention thereof by the applicant for a patent.

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

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(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

Claims 1, 3, 8, 9, 13-25, 31, 38, 47, 49 and 51-62 are rejected under 35 U.S.C. 102(b) as being anticipated by Huyghe et al. (Human Gene Therapy. 1995; 6: 1403-1416) in light of Kuchler for reasons of record.

In response to the issue of seeding density in section 1 bridging pages 12-13 of the response, applicant reasons that since the assumed seeding densities of Huyghe et al., provided by Mr. Gallagher's February 13, 2002 declaration, are found to be speculative and unsubstantiated, the Office's position must also be speculative and unsubstantiated. Both the Office and applicant appear to agree that Huyghe et al. do not discuss seeding densities. However, the Office does not and has not ventured to speculate or assume seeding densities that cannot be substantiated by Huyghe et al., as applicant has, since the reference does not discuss seeding densities. Applicant has not provided any argument or data sufficient to support the assumptions present in the declaration and the rejection is maintained.

Applicant asserts that Mediatech states that one should use 70% confluent cultures to ensure that they are in log phase. Applicant states that this teaching implies that cultures that are less confluent than 70% cannot be assured of being in log phase.

Applicant's arguments as well as a review of the reference have been fully considered, but are found to be unpersuasive. Mediatech correlates cells that are at least 70% confluent with log phase, see the top of the second column:

“Subculturing is usually performed during the log phase....[c]heck for cultures that appear to be at least 70% confluent.”

However, Mediatech does not differentiate cell confluency with the degree of log phase the cells may be in. For instance, Mediatech does not teach whether 70% confluency correlates to early, mid or late log phase. The reference only provides a general teaching that 70% confluency is log phase. Without any guidance provided by Mediatech regarding the percentage of confluency and the stage of log growth, it is maintained that a definite identification of which log phase a cell culture is in based on a percentage of confluency cannot be determined.

Regarding the teachings of Kuchler, applicant states that there is no correlation provided by the Office as to how or why the fibroblast suspension cultures of Kuchler and the 293 cells of Huyghe et al. are connected. However, applicant made the correlation when the reference was supplied to the Office for the general statement regarding typical lag phase and hours. Since the general teaching of Kuchler relied on by applicant as applying to the cells of Huyghe et al. is relevant, it is determined that all of the general teachings of Kuchler are relevant.

Applicant asserts that even if the teachings of Kuchler are relevant, the chart does not provide evidence that the cells of Huyghe et al. were infected after mid-log phase since the growth curve in Figure 3-1 shows the cells slightly before mid-log phase of growth.

A careful review of Figure 3-1 of Kuchler has been considered in view of applicant's remarks, but is found unpersuasive. If one were to draw a perfectly straight line parallel with the y-axis from the 60 hour point in Figure 3-1, the line would dissect the curve at exactly half-way, or mid-point, i.e. mid-phase. In addition, evidence that the cells of Huyghe et al. are infected

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after mid-log phase is not necessary since the claims only require that the cells are infected between mid-log phase and stationary phase.

Applicant also states that the cells depicted in Figure 3-1 of Kuchler have a faster doubling time and would arrive at mid-log much faster than the 293 cells of the instant application.

Applicant points to the declaration provided by Dr. Zhang as evidence that the doubling time of 293 cells is approximately 30 hours. Applicant assumes that if the doubling time of 293 cells is 30 hours, then the growth curve of Kuchler would be below mid-log.

Applicant's arguments as well as a review of the declaration have been fully considered, but are found unpersuasive. While the Quality Assurance Report states that the cell doubling time is approximately 30 hours, the Report does not indicate or imply which stage of log phase, i.e. early, mid or late log, the cells are in during this exponential growth phase. Therefore, from the evidence discussed in the previously, the rejection is maintained for reasons of record.

Claims 1, 3-9, 13-28, 30, 31, 38-49 and 51-62 are rejected under 35 U.S.C. 102(a) as being anticipated by Zhang et al. (WO 98/22588), as further evidenced by Wu et al. (US 6,689,600 B1) for reasons of record.

Claims 1, 3-9, 13-28, 30, 31, 38-49 and 51-62 are rejected under 35 U.S.C. 102(e) as being anticipated by Zhang et al. (US Patent No. 6,194,191 B1), as further evidenced by Wu et al. (US 6,689,600 B1) for reasons of record.

Applicant points to the declaration of Dr. Zhang and asserts that the instant invention was disclosed, but not claimed by the references cited. However, the entire disclosure of any reference disclosed to the public is available as prior art.

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Applicant also states that Dr. Zhang indicates in the declaration that the process was invented by him and the other co-inventors listed in the present application.

A review of the inventorship of both the instant application and the Zhang et al. references has been reviewed. It is determined that the inventive entity of both Zhang et al. references and the instant application are different since Shawn Gallagher is not listed as an inventor for either WO 98/22588 or US 6,194,191 B1. Since the statutes under 35 USC § 102 (a) and (e) require a description or disclosure by another and the Zhang et al. references and the instant invention have different inventive entities, it is maintained that both Zhang et al. references constitute prior art against the instant claims.

Applicant further asserts that Wu et al. is not available as prior art. It is not clear what applicant intends by "what one of ordinary skill in the art understood from the Zhang reference because the inventors on the Wu reference are the inventors of the Zhang reference."

Contrary to applicant's assertion, Wu et al. is available as prior art. The benefit of priority to the instant application is December 1, 1998 and the priority date granted to Wu et al. extends to a provisional application that was filed on November 16, 1998. In any case, the evidentiary teaching provided by Wu et al. is not required to antedate the instant filing date because it discloses evidence showing that the characteristic of when the cells of the Zhang et al. references are infected is at mid-log. See MPEP §§ 2131.01 and 2124.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person

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having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

Claims 10-12 and 29 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al. as applied to claims 1, 3, 8, 9, 13-25, 31, 38, 47, 49 and 51-62 above or Zhang et al. (WO 98/22588) or Zhang et al. US (6,194,191) as applied to claims 1, 3-9, 13-28, 30, 31, 38-49 and 51-62.

Applicant asserts that there is a contradiction between the seeding density being a crucial component of the log phase in the rejection of Huyghe et al. and that the instant seeding densities are merely a subjective determination.

In response, there is no discrepancy between the discussions. The seeding density is crucial for the log phase. Freshney, supplied to the Office by applicant, teaches this fact. It is also established by the teachings of the Kuchler that Huyghe et al. infect the cells at mid-log phase. Huyghe et al. do not disclose subjective factors, such as the condition of the cells before plating or the nature of the cell divisions, ect., but since the cells are required by the reference to be "about 50-60%" confluent at the time of infection, the exact number of cells plated by Huyghe et al. would have been a critical element in order to reach the required log phase by 60 hours.

Applicant also asserts that Huyghe et al. does not stand for reasons discussed previously. However, the reasons presented by applicant against Huyghe et al. are found to be insufficient.

Applicant also states that the Office has not explained how Huyghe et al. teach or suggest the adenovirus formulations of claim 29. Huyghe et al. teach a method of improving the quantity and/or purity of the recombinant virus obtained. Therefore, it would have been prima facie

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obvious to one of ordinary skill to test any one of the properties listed to ensure a good yield of adenovirus.

Applicant maintains that Zhang et al. is not prior art. However, this assertion is rendered moot since each Zhang et al. reference is a different inventive entity from the instant application.

Claims 2 and 50 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al. as applied to claims 1, 3, 8, 9-12, 13-25, 29, 31, 38, 47, 49 and 51-62 above or Zhang et al. (WO 98/22588) or Zhang et al. US (6,194,191) as applied to claims 1, 3-31, 38-49 and 51-62 and further in view of Graham et al. (C31 of IDS) and Leu et al. (6,194,210 B1) for reasons of record.

Applicant argues that the statement by the Office that the teachings of Leu et al. are applicable to the general viral propagation art does not make it so. Applicant asserts that the discussion about other viruses in Leu et al. is referring to viruses that can be propagated in hosts, not that they can infect cells at late-log phase. Applicant also argues that it has not been explained how Leu et al. can be combined with Huyghe et al., i.e. whether infecting cells at different phases of growth are beneficial to achieve increased production of adenovirus. Applicant also argues that there does not appear to be a reason why the skilled artisan would turn to the teachings of Leu et al. Applicant cites case law for support.

In response, the Office does not make the statement about Leu et al. without explicit support from the reference, see column 5, lines 29-32, column 11, lines 18-column 12, line 9 and claims 1 and 4. The list of various viruses discussed by Leu et al. apply to the method of producing large quantities of virus, see claims 1 and 4. Making large quantities of virus, explicitly claimed by Leu et al., would have been the motivating factor for one of ordinary skill

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in the art to modify the teachings of Huyghe et al. by infecting cells at late-log phase, see column 11, lines 18-column 12. Obviousness can only be established by combining or modifying the teachings of the prior art to produce the claimed invention where there is some teaching, suggestion, or motivation to do so found either in the references themselves or in the knowledge generally available to one of ordinary skill in the art (emphasis added). See *In re Fine*, 837 F.2d 1071, 5 USPQ2d 1596 (Fed. Cir. 1988) and *In re Jones*, 958 F.2d 347, 21 USPQ2d 1941 (Fed. Cir. 1992). In this case, the motivation of producing large quantities of virus is found explicitly within the teaching of Leu et al. Leu et al. accomplish this by infecting cells in late-log phase. One of ordinary skill in the art at the time the invention was made would have been had a reasonable expectation of growing the adenovirus of Huyghe et al. or either Zhang et al. reference with the cell culture method steps taught by Leu et al. because Leu et al. teach that a wide range of viruses may be propagated to generate vaccines using the method steps, see column 5, lines 29-32 and Graham et al. teach infecting cells at 80-90% (late-log phase) with adenovirus, see section 3.1.2 on page 117. Therefore, Graham et al. clearly demonstrate that the teachings of Leu et al. are applicable to adenovirus infection in cells at late-log phase of growth. Therefore, the rejections are maintained for reasons of record.

With respect to Leu et al. and Graham et al. in view of either Zhang et al. reference, applicant cites the declaration of Dr. Zhang. However, since this declaration does not obviate the primary rejections of record for reasons discussed above.

Claims 26-28 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al. as applied to claims 1, 3, 8, 9-12, 13-25, 29, 31, 38, 47, 49 and 51-62 above, and further in view of Graham et al. (C7) for reasons of record.

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Applicant argues that Graham et al. do not cure the deficiencies of Leu et al., Huyghe et al. or either Zhang et al. reference. Applicant also argues that Graham et al. do not provide a motivation to combine Leu et al., Huyghe et al. or either Zhang et al. reference.

Applicant's arguments have been fully considered, but are found unpersuasive since there are no deficiencies for Graham et al. to cure. Motivation(s) for combining Huyghe et al. or either Zhang et al. reference with Leu et al. are supplied in the previous rejection.

Claims 4, 30, 39-46 and 48 are rejected under 35 U.S.C. 103(a) as being unpatentable over Huyghe et al. as applied to claims 1, 3, 8, 9-12, 13-25, 29, 31, 38, 47, 49 and 51-62 above, and further in view of Garnier et al. (C26) and Spier et al. (C35 of the IDS) for reasons of record.

Applicant maintains that the 293 cells of Huyghe et al. are infected at early log phase. However, this assertion remains unsupported.

Applicant also states that Garnier et al. is only concerned with an increase in the production of heterologous proteins and not adenovirus titers. Applicant also states that the rejection is silent with respect to when infection of the cells took place. Applicant concludes that Spier et al. do not even mention adenovirus and therefore, does not provide a motivation for the combination of references cited.

Applicant's arguments as well as a review of the reference have been fully considered, but are found unpersuasive. The scale-up method "to improve the volumetric yield of the [adenovirus] recombinant protein production system" quoted by applicant necessarily requires an increased yield of adenoviruses carrying the heterologous protein. An increase of protein produced/or expressed by the adenovirus necessarily means that more adenovirus is present in greater quantities, i.e. a volumetric yield. The teachings of Garnier et al. are not required to

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teach when infection took place because this teaching is supplied by Huyghe et al. It is established that Huyghe et al. teach infection at the mid-log phase. Spier et al. review conventional techniques used in the virus propagation art. One of ordinary skill would have been motivated to use a conventionally applied culture system, described by Spier et al. in the method and system of Huyghe et al. and Garnier et al. One of ordinary skill in the art at the time the invention was made would have had a reasonable expectation of success in using any of the culture systems of Spier et al. in the method of Garnier et al. and Huyghe et al. because Garnier et al. use a bioreactor system to propagate large quantities of adenovirus and Spier et al. review various types of bioreactor systems.

Therefore, the invention as a whole would have been prima facie obvious to one of ordinary skill in the art, absent unexpected results to the contrary.

Conclusion

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

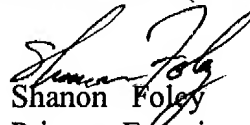
A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

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Any inquiry concerning this communication or earlier communications from the examiner should be directed to Shanon Foley whose telephone number is (571) 272-0898. The examiner can normally be reached on M-F 10:00 AM - 6:30 PM.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, James Housel can be reached on (571) 272-0902. The fax phone number for the organization where this application or proceeding is assigned is 703-872-9306.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).


Shanon Foley
Primary Examiner
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